EFFECTS OF GLUTATHIONE PEROXIDASE 1, GLUTATHIONE PEROXIDASE 4 AND COPPER, ZINC-SUPEROXIDE DISMUTASE GENE KNOCKOUTS ON ENZYME ACTIVITIES, BODY SELENIUM AND RESISTANCE TO OXIDATIVE AND NITROSATIVE STRESSES

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ABSTRACT

This thesis addresses two studies, 1) characterization of a glutathione peroxidase 4 (GPx4) haploid insufficient (+/-) mouse and 2) effects of different levels of glutathione peroxidase 1 (GPx1) and Cu, Zn - superoxide dismutase 1 (SOD1) gene dosage on enzyme expression and responses to paraquat (PQ), diquat (DQ) and/or acetaminophen (AP) toxicity. These studies are united in a general way by addressing response to oxidative stress with altered antioxidant enzyme expression and in a more specific way by involvement of two selenium (Se) containing glutathione peroxidases (GPx1 and GPx4) and SOD1. All three of these enzymes are believed to be involved in *in vivo* metabolism of reactive oxygen species (ROS).

GPx4 is structurally and functionally unique among selenoperoxidases since it functions as a monomer and is able to metabolize phospholipid hydroperoxides. GPx4 is more resistant to Se depletion than other selenoperoxidases and deletion of both GPx4 alleles is embryonic lethal, indicating an important role that is not yet fully understood.

Experiment 1 examined the effects of deletion of one *gpx4* allele at baseline and with a ROS challenge. GPx4+/- and wild-type (WT or GPx4+/+) mice were injected with 24 mg/kg body weight of the ROS generator PQ or phosphate buffered saline (PBS) control and sacrificed 4 h later. GPx4+/- mice had decreased GPx4 activity in lung, liver, kidney and testis, from 24 to 39% ($P < 0.05$) lower activity than WT. GPx4+/- mice had a 34% ($P < 0.05$) decrease in testis Se concentration. GPx4+/- had no effect on Se concentration, protein carbonyl formation (measure of oxidized protein) or GPx1 activity in other tissues or GPx3 and alanine aminotransferase (ALT) activity in plasma. In summary, deletion of one *gpx4* allele demonstrated a range of effects on GPx4 activities and Se concentrations, but did not affect

susceptibilities to pro-oxidant-induced protein oxidation in various tissues of mice.

SOD1 and GPx1 are often considered to detoxify ROS in the cytosol. SOD1 produces hydrogen peroxide from superoxide which GPx1 reduces to water. A dramatic increase in ROS lethality has been found in cells and mice lacking GPx1 or SOD1 (GPx1-/- or SOD1-/-) whereas GPx1-/- hepatocytes are protected against reactive nitrogen species (RNS) induced cell death.

Experiment 2 compared responses of mice with various gene dosages of both SOD1 and GPx1 in a 72 h survival trial to the ROS generator DQ (25 mg/kg body weight) or the putative *in vivo* RNS generator AP (600 mg/kg body weight). Although this experiment was limited by sample size and group death rates, some general trends emerged. The SOD1 knockout allele decreased AP-induced mortality and increased DQ-induced mortality ($P < 0.05$). The GPx1 knockout allele increased mortality from both AP and DQ ($P < 0.05$). In combination, mice with only one functional copy in total of SOD1 and GPx1 (GPx1+/-|SOD1-/- or GPx1-/-|SOD1+/-) had significantly decreased DQ survival time but unchanged AP survival time ($P < 0.05$). AP-treated GPx1+/-|SOD1+/+ mice died significantly earlier than control (<40 h) but GPx1+/-|SOD1+/- survival time was not significantly different from control (>72 h). SOD1 knockout was also associated with 28 to 34% decreases in GPx1 activity, ($P < 0.05$; +/+ vs. +/- and $P = 0.078$ +/+ vs. -/-), depending on SOD1 copy number). Plasma ALT peaked between 20-40 h (11740 ± 1074) with AP treatment and <20 h with DQ treatment (2129 ± 537) but was unchanged in all surviving mice.

In summary, there are a variety of responses to antioxidant enzyme knockouts and not all are detrimental to defense against oxidative stress. In Experiment 1, *gpx4* deletion reduced tissue GPx4 activity and testis Se without

increased susceptibility to PQ toxicity. In Experiment 2, SOD1 knockout was associated with high resistance to AP toxicity, high susceptibility to DQ toxicity and decreased GPx1 activity while GPx1 knockout was associated with increased AP and DQ mortality. Overall, the effect of SOD1 knockout on resistance to AP lethality was greater than the effect of GPx1 knockout on AP lethality.

BIOGRAPHICAL SKETCH

Michael Salvatore Scimeca was born on September 11, 1975 in Kingston, NY and grew up in New York State's Mid-Hudson Valley. He graduated from Poughkeepsie Day School in 1993 and attended Cornell University. In 1997, he graduated with a BS degree in Animal Science. That same year he moved to Boston to take a job as a research technician at Harvard Medical School / Massachusetts Eye and Ear Infirmary studying hereditary retinal diseases. In 1999 he married his college sweetheart, Clay Chiment and in the following year, took a job in the Division of Comparative Medicine at the Massachusetts Institute of Technology studying Helicobacter and gastrointestinal cancer. In fall of 2001, he returned to the Cornell University Department of Animal Science to pursue his graduate studies. His daughter, Laia, was born on April 5, 2005.

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CHAPTER 1:

LITERATURE REVIEW

Introduction

In the process of normal aerobic metabolism, reactive oxygen species (ROS) are formed. Enzymatic systems are a very important means by which cells can maintain these potentially damaging molecules within concentrations needed for normal cellular function. At normal concentrations, ROS such as superoxide, hydrogen peroxide and lipid hydroperoxides are able to regulate activities of kinases, transcription factors and apoptotic factors in addition to contributing to the normal function of many other metabolic and signaling systems. Chronically increased levels of ROS can contribute to pathological states including cancer and cardiovascular disease (Finkel, 1998; Rhee, 1999; Thannickal and Fanburg, 2000; Nomura et al., 2001) while acute increases in ROS can lead to massive damage of biomolecules (e.g. protein, DNA and lipids) and consumption of reducing molecules (e.g. NADPH) leading to cellular apoptosis or necrosis and, on a whole animal scale, death (Bus et al., 1974; Smith, 1977; Witschi et al., 1977; Cagen and Gibson, 1977; Keeling and Smith, 1982; Burk, 1991; Sunde, 1994; Ho et al., 1997; Berlett and Stadtman, 1997; Cheng et al., 1998; De Haan et al., 1998; Cheng et al., 1999). Selenium-dependent glutathione peroxidases 1 and 4 and copper, zinc-dependent superoxide dismutase 1 are three important micronutrient dependent enzymes in coping with oxidative stress (McCord and Fridovich, 1969; Flohe et al., 1973; Rotruck et al., 1973).

Selenium

Although the first report of Se in mammalian tissue was in 1916 (Gassmann, 1916), this finding was considered to be an artifact (See: *Gmelins*

Handbuch der Anorganischen Chemie, Selen, Vol. 1, Part A, p. 24., 1953) and Se was understood to be toxic. Many years passed before Se was finally recognized as an essential nutrient in bacteria by Pinsent et al., (1954) and in rats by Schwartz and Foltz (1957). This delay in the discovery of Se as a nutrient is in part due, as later work showed, to the ability Se and vitamin E to spare one another's requirement for prevention of some signs of deficiency (Thompson and Scott, 1969; Scott, 1980). Careful observation showed that some of these symptoms responded specifically to one or the other of these nutrients (Harris et al., 1958).

An example of the importance of selenium in the human diet, Keshan disease, an endemic juvenile onset cardiomyopathy, has been traced to the low soil selenium content in a region of China, leading to low Se intake and association with low body Se content (Keshan disease research group, 1979a). A study of Se supplementation followed these discoveries which showed that it helped to prevent the disease (Keshan disease research group, 1979b). Another interesting feature of Keshan disease is that it cannot be explained by Se deficiency alone. The cardiomyopathy has been associated with the virus Coxsackie B4, which mutates under conditions of low Se to become pathogenic (Levander and Beck, 1997). Among other species, Se deficiency is associated with a number of different symptoms. In rats, Se deficiency is associated with sparse hair coat, poor growth, poor sperm motility and cataracts (see Levander et al., 1995 for review). In ruminants, Se deficiency can cause white muscle disease (a nutritional muscular dystrophy) (Muth et al., 1958), dystrophic tongue, heart failure and retained placenta. Se deficiency is associated with reduced serum selenium, increased AST activity and white muscle disease in horses. Deficiencies of Se and vitamin E can cause sudden death of young, rapidly growing pigs and a deficiency of

selenium in growing chickens causes exudative diathesis (a weeping edema of the skin with easy bruising) (see The Merck Veterinary Manual, Eighth Edition [2003] for review).

Selenoproteins

The vast majority of mammalian selenoproteins incorporate Se in the form of the 21st amino acid, selenocysteine (Bock, 2001; Hatfield and Gladyshev, 2002). In many cases, selenium is present at a catalytic center of an enzyme responsible for redox reactions (Stadtman, 2000; 2001). In selenoprotein mRNA, the stop codon UGA encodes SeCys insertion, requiring a specialized group of transcription factors to translate it properly (see Berry et al. 2001 and Copeland, 2003 for review). The process of SeCys incorporation is highly selenium dependent, requiring both cis and trans acting components that lead to a hierarchy of Se-protein expression (Allan et al., 1996; Sunde, 2001). Se deficiency often results in a lack of selenoprotein expression through altered mRNA stability and protein translation (Chu et al., 1990; Allan et al., 1999).

Selenocysteine incorporation requires a SeCys charged tRNA containing the UCA codon (Diamond et al., 1981; Leinfelder et al., 1988). The SeCys tRNA has methylated and non-methylated isoforms, indicating a possible mechanism for mediating the hierarchy of Se incorporation into protein (Jameson and Diamond, 2004)

Although the eukaryotic synthesis of SeCys tRNA is not yet fully characterized, a partial picture of the process has emerged. Selenocysteine is synthesized on a tRNA initially charged with serine (Carlson et al., 2001), which is phosphorylated to form a phosphoseryl tRNA. An activated selenium group (most likely monoselenophosphate, formed by a selenophosphate

synthetase) is then exchanged for the phosphate group, completing the synthesis of SeCys tRNA (see Review by Hatfield and Gladyshev, 2002).

In order to incorporate SeCys at the UGA codon, a cis-acting selenocysteine insertion sequence (SECIS) is located in the 3' untranslated region (3'UTR) of the mRNA (Berry et al., 1991). The sequence of SECIS is not highly conserved among eukaryotes but they are structurally similar across species, forming a stem-loop (Martin and Berry, 2001). The SECIS binding protein 2 (SBP2) which has been shown to bind with SECIS, is able to interact with ribosomes (Copeland et al., 2001). It is believed that these characteristics allow SBP2 to alter the ribosomes reading of the genetic code such that UGA leads to SeCys incorporation (Copeland et al., 2001).

A recent paper by Kryukov et al., (2003) reported 25 distinct putative selenoproteins, identified by a computer algorithm based on the presence of the SeCys codon and a SECIS element. Although not all of these may be active genes, it is exciting to consider these potential subjects of selenoprotein research next to the known and characterized selenoproteins (n=14, based on Lei, 2001).

Among the well characterized selenoproteins (Burk and Hill, 1993; Sunde, 1994; Stadtman, 1996; Flohe et al., 1997), the glutathione peroxidases form the largest single family. Included in this group are cellular or classical glutathione peroxidase (GPx1), gastrointestinal or GI glutathione peroxidase (GPx2), plasma or extracellular glutathione peroxidase (GPx3) and phospholipid hydroperoxide glutathione peroxidase (GPx4). The recently discovered glutathione peroxidase 6 is present in adult olfactory epithelium, however, while the human isoform does contain selenium, the isoform found in mouse does not. The iodothyronine deiodinases, types 1, 2 and 3 (ID1, ID2 and ID3) regulate thyroid hormones by catalyzing their deiodination (Berry et

al., 1991; Croteau et al., 1996; Salvatore et al., 1995). Another group of selenoproteins with antioxidant functionality are the thioredoxin reductases which reduce the thiol containing small protein thioredoxin (Tamura and Stadtman, 1996; Gromer et al., 1998; Sun et al., 1999). The ATP dependent activation of selenium during selenocysteine synthesis is catalyzed by the selenoprotein Selenophosphate synthetase (Kim et al., 1997). Accounting for approximately 65% of plasma selenium, Selenoprotein P (Sel P) also has some antioxidant properties but its role is still unclear (Saito et al., 1999). Containing 10 SeCys residues, Sel P is also the only known selenoprotein to contain more than one SeCys residue (Hill et al., 1991). It has also been found to have multiple isoforms forms in the rat (Himeno et al., 1996; Chittum et al., 1996). Selenoprotein W (Sel W) is a small (10kDa) selenoprotein expressed in all tissues. The physiological role of Sel W is still unknown but it may be related to white muscle disease (a disease of Se deficiency) in animals (Vendeland et al., 1993; 1995). A third selenoprotein whose role is as yet unknown is the 15kDa selenoprotein (15-kD Sel) which was discovered in human T cells and is expressed ubiquitously (Gladyshev et al., 1998).

Glutathione and the Glutathione Peroxidases

The selenium dependent glutathione peroxidases share certain characteristics, including the presence of a single SeCys residue per monomer and the ability to reduce peroxides using glutathione (GSH) as a cofactor, producing a cognate alcohol and glutathione disulfide (GSSG). Substrate specificity varies among the Se-GPx family; for example, only GPx4 is able to efficiently reduce phospholipid hydroperoxides. There is also structural variation among these proteins with GPx1, 2 and 3 functioning as tetramers of approximately 90kDa while GPx4 functions as a monomer of approximately 20

kDa . Expression patterns also distinguish the glutathione peroxidases. GPx1 is expressed ubiquitously but is especially high in the liver and kidney. GPx2 expression is primarily in the gastrointestinal tract and GPx3 is expressed extracellularly as a glycoprotein. The expression of GPx4 is also ubiquitous but expression is particularly high in the testes (see Lei, 2001 for review).

Glutathione

Glutathione is a thiol containing tripeptide (Glu-Cys-Gly) which serves as a cofactor for the glutathione peroxidases. Glutathione and other low molecular weight thiols have the advantage in redox reactions of being easily oxidized and regenerated. Due to these characteristics, glutathione can play an essential role in many biochemical and pharmacological reactions (Mates, 2000; Locigno and Castronovo, 2001; Paolicchi et al., 2002). Some of the important roles of glutathione are: reduction or inactivation of ROS and RNS by formation of glutathione disulfide (GSSG) and conjugation of reduced glutathione (GSH) with a xenobiotic and subsequent elimination as a mercapturic acid (Berlett and Stadtman, 1997; Hayes et al., 1999; Strange et al., 2001). The functions of the glutathione system in redox balance and in xenobiotic detoxification demonstrate the importance of understanding this system in a physiological context and make it an interesting potential target for genetic and therapeutic manipulation.

Glutathione Peroxidase 1

Glutathione Peroxidase 1 (EC: 1.11.1.9, GPx1), also known as cellular or “classic” glutathione peroxidase was the first identified mammalian selenoprotein (Rotruck et al., 1972) in a report of hydrogen peroxide dependent hemolysis which was not prevented by glucose in Se deficient rats.

This discovery was foreshadowed by the work of Mills (1957) and Schwarz and Folz (1957) who discovered GPx protection of hemoglobin against oxidation and the essentiality of Se in preventing rat liver necrosis. Additionally, Mills et al., (1959, 1960) identified GPx1 as a distinct peroxidase from others already known since its activity could not be inhibited by azide or cyanide.

Flohe et al., (1972) identified GPx1 as a 23 kDa protein containing one Se atom which functions as a homotetramer of 88 kDa. In the mouse, the GPX1 gene is 5.2 kb in length with two exons and one intron (Chambers et al., 1986; Ho et al.,1997). Mammalian GPx1 proteins are approximately 201 amino acids in length with a selenocysteine at position 47 (Sunde, 1994). Most GPx1 activity (75%) is found in the cytosol with the remaining 25% of activity in the mitochondria (Flohe, 1989). Although GPx1 activity is expressed in most tissues of rodents, the liver and kidney have the highest levels of activity (Lei et al., 1995; Bermano et al., 1995; Cheng et al., 1998).

Expression of GPx1 varies widely and in rapid fashion to changes in bodily Se status (Cheng et al., 1997). These Se dependent expression patterns show a range of effect among the Se- glutathione peroxidases. For example, by 130 days of Se depletion, mouse liver GPx1 activity falls to near zero while 40% of GPx4 activity remains (Weitzel et al., 1990). Activity and mRNA follow the same pattern in rats with 1 and 6% of Se adequate levels remaining, respectively while liver GPx4 activity is reduced to about 40% and mRNA levels are nearly unchanged (Lei et al., 1995).

For some time, the *in vivo* role of GPx1 was unclear. Although studies in cells supported the function of GPx1 in protecting against ROS (Mirault et al., 1991; Geiger et al., 1993) there was disagreement about whether GPx1 filled the same role in vivo (Burk et al., 1980; Mercurio and Combs, 1986a; 1986b;

Burk et al., 1995). Based on its response to Se deficiency relative to other Se proteins, a major alternative hypothesis to GPx1 as an antioxidant is a role in Se homeostasis (Burk, 1991; Sunde, 1994). In support of this, the GPx1 knockout mouse appeared to have no phenotypic defects and showed no increased sensitivity to hyperoxia (Ho et al., 1997). However, work by Cheng et al., (1997) found that GPx1 knockout in mice had no effect on other selenoperoxidases (e.g. GPx3, GPx4) under conditions of Se adequacy and depletion, indicating that GPx1 was not acting as a buffer of bodily Se. More convincingly, Cheng et al., (1998) used the GPx1 knockout mouse model to show that GPx1 mediated selenium's protection against acute oxidative stress. Based on this work, which is supported by the findings of others (De Haan et al., 1998; Fu et al., 1999a; 1999b), there is solid evidence for the role of GPx1 as a protector against acute oxidative stress. An additional related finding by Fu et al., (2001) is that while GPx1 knockout increases hepatocyte sensitivity to the ROS produced by the superoxide generator diquat, it protects against peroxynitrite induced apoptosis.

Glutathione Peroxidase 2

Gastrointestinal glutathione peroxidase, (GI-GPx or GPx2) is closely related to GPx1 in terms of structure and substrate specificity but differs in tissue distribution, GPx2 being almost exclusively expressed in the GI tract, specifically in the crypts of the intestinal epithelium (Utsunomiya et al., 1991; Chu et al., 1993; Esworthy et al., 1997). In addition, the mRNA of GPx2 is much more resistant to Se deficiency than that of either GPx1 or GPx3, suggesting an important physiological role (Weitzel et al., 1990; Chu et al., 1997; Wingler et al., 1999; Brigelius-Flohe, 1999). Mice lacking both GPx1 and GPx2 (GPx1/2-KO) exhibit severe ileocolitis at a early age whereas mice that

have one intact copy of GPx2 have a much lower incidence of inflammatory bowel symptoms (Thompson et al., 1998; Esworthy et al., 2001; 2003). GPx1/2-KO mice also have microflora-associated cancers in the lower GI tract with 25% of mice colonized with *Helicobacter* species developing ileal and colonic tumors (Chu et al., 2004).

Glutathione Peroxidase 3

Plasma glutathione peroxidase or GPX3 is an extracellular glycosylated enzyme (Bjornstedt et al., 1994; Tham et al., 1998) which can reduce hydrogen peroxide, lipid hydroperoxides and phospholipid hydroperoxides, although with slower catalytic rates than other selenoperoxidases (Esworthy et al., 1991; Yamamoto and Takahashi,1993). Since it functions as a tetramer, has a similar molecular weight and similar substrate specificty, GPx3 was originally considered to be the same as GPx1, but later found to have different biochemical characteristics and distinct regulation (Cohen et al., 1985; Takahashi and Cohen, 1986; Cohen and Avissar, 1994). The cDNA of GPx3 contains 5 exons, is approximately 10kb in length (Yoshimura et al., 1994) and has 44% homology with human GPx1 (Yoshimura et al., 1994). It encodes 226 amino acids with a SeCys at position 73 (Takahashi et al., 1990) has a molecular mass of 92kDa (Cohen and Avissar, 1994). GPx3 is primarily produced in the kidney and exported as a glycoprotein into plasma (Yoshimura et al., 1991). Although it is able to reduce peroxides and phospholipid hydroperoxides in vitro (Esworthy et al., 1993), the concentration of plasma GSH is believed to be too low for this function (Cohen and Avissar, 1994) and therefore it is hypothesized to protect against peroxides in the renal extracellular space (Maser et al., 1994), the fluid of lung epithelium and

interstitial space (Avissar et al., 1996) and the intestinal intercellular space (Tham et al., 1998).

Glutathione Peroxidase 4

Phospholipid hydroperoxide glutathione peroxidase or GPx4 (EC 1.11.1.12) was discovered by Ursini et al. (1982) as a selenoprotein in pig liver extract with the ability to protect cellular lipids against peroxidation and to reduce phosphatidylcholine hydroperoxides. Being distinct from cellular glutathione peroxidase-1 (GPx1) (Schukelt et al., 1991), GPx4 is expressed as three isoforms with alternate start codons and exons: a 23 kDa form (with a 27 amino acid mitochondrial targeting sequence that is later cleaved), a 20 kDa non-mitochondrial form and a 34 kDa sperm nucleus form (with an alternate first exon) (Pushpa-Rekha et al., 1995; Arai et al., 1996). Compared with other selenoperoxidases, GPx4 shares approximately 30 to 40 % nucleotide identity (Imai and Nakagawa, 2003). The enzyme functions as a monomer rather than a tetramer (as in the case of other GPx proteins), and it is the only GPx that is able to reduce phospholipid hydroperoxides (Ursini et al., 1985). Nutritionally, GPx4 is much more resistant to dietary Se deficiency than the other GPx enzymes, particularly GPx1. When liver GPx1 activity and protein are reduced to nearly zero in selenium-depleted rodents (Weitzel et al., 1990; Lei et al., 1995; Bermano et al., 1996), liver GPx4 activity maintains approximately 20% of the selenium adequate levels (Weitzel et al.,1990; Thompson et al., 1998).

GPx4 was initially suggested as an important antioxidant enzyme (Ursini et al., 1985; Wang et al., 2001; Yant et al., 2003; Ran et al., 2003; 2004). Yant et al. (2003) found that GPx4 hemizygous mice were more sensitive to gamma irradiation than WT mice, presumably due to induction of free radicals. In addition, they found that GPx4 +/- murine embryonic fibroblasts were more

sensitive to an assortment of pro-oxidants. However, this function of GPx4 has not been tested in vivo.

Yant et al., (2003) found that a full knockout of *gpx4* in mice is lethal at embryonic day 7.5 and postulated an essential function of GPx4 in development. It is well known that GPx4 protein and activity are very high in testes (Weitzel et al., 1990; Roveri et al., 1992). Ursini et al. (1999) reported that GPx4 is involved in sperm maturation and serves a structural role in the sperm tailpiece in an oxidatively cross-linked state. In addition, Foresta et al. (2002) found a causative link between selenium deficiency and male infertility and Behne et al. (1996) found that Se deficient rats produce abnormal sperm and show decreased fertility. Conrad et al. (2000) made a preliminary report of abnormal sperm and focal necrotic lesions in the testes of mice chimeric for a full knockout of GPx4. Due to its important functions in sperm maturation and high levels in the testes, it has been assumed that the detrimental effects of selenium deficiency on male reproductive function are mediated through GPx4. However, the relative portion of total tissue Se in the form of GPx4 protein and the effect of GPx4 expression on the expression of other selenoperoxidases are unclear and could not be determined using conventional selenium-deficient animal models.

Copper, Zinc and the Superoxide Dismutases

Copper and zinc serve as components of proteins important for cytosolic antioxidant defense. A primary example of this is copper, zinc superoxide dismutase (SOD1, EC 1.15.1.1), which catalyzes the dismutation of hydrogen peroxide to oxygen and hydrogen peroxide which is then reduced to water by the action of the selenoprotein glutathione peroxidases (see **Figure 1.1** and **Figure 1.2**) (McCord and Fridovich, 1969; Flohe et al., 1973; Rotruck et al.,

1973). In addition to its activity in oxidant defense, copper, as a redox active metal can also participate in the production of free radicals by interaction with thiols (e.g. reduced glutathione) and oxygen. In situations of high intracellular hydroperoxide concentrations, copper (and more classically, iron) also has the potential to participate in Fenton reactions (**Figure 1.3**) which produce hydroxyl radicals (Oshino et al., 1973). Due to its potentially damaging reactivity, there are high levels of copper scavenging proteins in the cell, maintaining free copper ion concentrations at near zero (Rae et al., 1999). Among these scavenging proteins are the metal binding proteins, the metallothioneins, the expression of which can be induced by copper (Murata et al., 1999). Copper plays a vital role as a co-factor for a number of metalloenzymes including:

- Cu/Zn superoxide dismutase (antioxidant defense),
- cytochrome c oxidase (mitochondrial respiration),
- lysyl oxidase (formation of connective tissue),
- tyrosinase (melanin synthesis)
- ceruloplasmin (iron homeostasis) (Pena et al., 1999; Shim and Harris, 2003)

Many of the symptoms associated with copper deficiency are a consequence of decreased activity of copper-dependent enzymes (Prohaska, 1991; Milne and Nielsen, 1996; Turnlund et al.,1997; Kehoe et al., 2000) although overt copper deficiency is rare in humans.

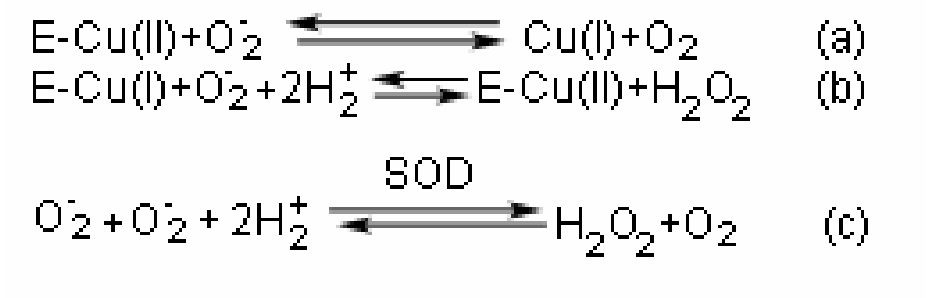

Figure 1.1. The established function of Superoxide Dismutases. SOD function proceeds by two sequential reactions with superoxide giving an overall reaction of **(c).** *from* Liochev et al., (2003)

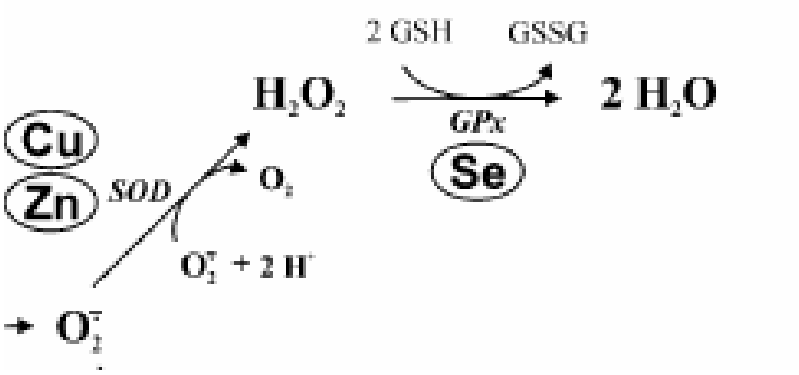

Figure 1.2. The Superoxide Dismutase – Glutathione Peroxidase ROS Pathway. Modified from Klotz et al., (2003)

$$Fe^{2+} + H_2O_2 > Fe^{3+} + OH^{\circ} + OH^{-}$$

Figure1.3. The Fenton reaction. This is the iron-salt-dependent decomposition of hydrogen peroxide, generating the highly reactive hydroxyl radical, possibly *via* an oxoiron(IV) intermediate. *from:* Compendium of Chemical Terminology, page 1274

Originally known as erythrocuprein, copper, zinc superoxide dismutase (SOD1) (EC 1.15.1.1) was the first SOD identified (McCord and Fridovich, 1969). Now three distinct superoxide dismutases are known in mammals, with their genomic structure, cDNA, and proteins described. Two of these SOD

isoforms have copper and zinc in their catalytic center, cytosolic SOD1 and extracellular SOD3. SOD1 is a homodimer of about 32kDa (Chang et al., 1988; Keller et al., 1991; Crapo et al., 1992; Liou et al.,1993). First detected in human plasma, lymph, ascites, and cerebrospinal fluids (Marklund et al., 1982, 1986), SOD3 functions as a homotetramer of 135 kDa (Marklund, 1982). The third SOD isoform, MnSOD or SOD2, contains manganese (Mn) as a cofactor and has been localized to the mitochondria of aerobic cells (Weisiger and Fridovich, 1973).

The genomic sequence for SOD1 shows striking similarity among species being made up of five exons and four introns (Levanon et al.,1985; Benedetto et al., 1991; Hsu et al., 1992; Kim et al., 1993) The promoter region of human SOD1 has putative binding sites for the transcription factors NF1, Sp1, AP1, AP2, GRE, HSF, and NF-κB (Kim et al., 1994). A number of different mutations in SOD1 have been associated with amyotrophic lateral sclerosis (ALS) or Lou Gehrig's disease (Rosen et al., 1993). However, these seem to be "gain of function" mutations, altering SOD1's substrate affinity, decreasing its zinc binding ability or causing increased enzyme aggregation in motor neurons (Chou et al., 1996; Wiedau-Pazos et al., 1996; Bruijn et al., 1998; Estevez et al., 1999). In further support of this, deletion of SOD1 in knockout mice does not cause any motor neuron abnormalities (Reaume et al., 1996).

SOD1 catalyzes the reactions depicted in **Figure 1.1**, producing hydrogen peroxide and molecular oxygen from superoxide and protons with redox cycling of copper (McCord and Fridovich, 1969; Liochev and Fridovich, 2003). Hydrogen peroxide can then be detoxified by the glutathione peroxidases (See **Figure 1.2**). However, in the case of an imbalance of superoxide production and SOD function, superoxide can accumulate in the

cell. Likewise, when hydrogen peroxide production outpaces GPx activity, a buildup can occur. When this happens, there is opportunity for these toxic metabolites to damage biomolecules. Hydrogen peroxide can undergo the Fenton reaction (See **Figure 1.3**) to produce the very highly reactive hydroxyl radical (See Compendium of Chemical Terminology, p.1274).

Ho et al., (1998) generated mice deficient in SOD1 by gene-targeting technology. These mice lacked both SOD1 mRNA expression and SOD1 enzyme activity in all tissues examined. These SOD1 knockout (SOD1-/-) mice developed normally and appeared healthy and even showed no change in sensitivity to hyperoxia (Ho et al., 1998). However, the SOD1-/- mice did show increased sensitivity to acute paraquat toxicity, myocardial ischemia/reperfusion injury, and *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurodegeneration (Ho et al., 1998; Yoshida et al., 2000; Zhang et al., 2000). Another significant finding by Ho et al., (1998) is that SOD1-/- female mice have greatly reduced fertility compared to those with one (SOD1+/-) or two (SOD1+/+) functional copies of SOD1 although the mechanism for this remains unexplained.

Acetaminophen: inducer of reactive nitrogen species and oxidative stress

Although it is considered very safe at normal doses, the analgesic and antipyretic acetaminophen (AP) can produce a potentially fatal centrilobular hepatic necrosis when an overdose is taken (Prescott, 1980). Overdose of AP is one of the most common pharmaceutical product poisonings in the United States (Litovitz et al., 2002). Although the precise mechanisms of hepatocyte death are not well understood, necrosis, rather than apoptosis is recognized as the mode of cell death (Lawson et al.,1999; Gujral et al., 2002).

Acetaminophen is metabolically activated by cytochrome P450 by a direct two-electron oxidation to form *N*-acetyl-*p*-benzoquinoneimine (NAPQI). NAPQI is then able to covalently bind to protein (Mitchell et al., 1973; Dahlin et al., 1984), potentially altering or eliminating function. Several cytochromes, primarily P450 2E1, (Patten et al., 1993; Thummel et al., 1993; Chen et al., 1998) have been reported to be involved in the production of NAPQI. Detoxification of NAPQI is thorough conjugation with GSH to form an AP-GSH conjugate which can then be eliminated. However, in the case of a toxic dose, total hepatic GSH is depleted and NAPQI binds to thiol groups on proteins forming AP-protein adducts (Mitchell et al., 1973). The progression of toxicity to produce hepatic cell death following protein adduct formation is poorly understood. It is hypothesized that AP adduct formation with important proteins can disrupt cellular function, leading to cell death. Primary cellular targets may be mitochondrial proteins and proteins involved in cellular ion homeostasis (Nelson, 1990).

Oxidative stress may also contribute to AP toxicity since during formation of NAPQI by cytochrome P450, the superoxide anion is formed. Subsequently, through SOD activity, hydrogen peroxide is formed (Dai and Cederbaum, 1995). In support of this, Nakae et al., (1990) reported that administration of encapsulated superoxide dismutase decreased the toxicity of acetaminophen in the rat. It is postulated that under conditions of depleted GSH and consequent decreased GPx activity, hydrogen peroxide concentrations could increase and Fenton-type peroxidation reactions could occur (Nakamura et al., 1974; James et al., 2003).

Nitrotyrosine, an excellent biomarker of peroxynitrite formation (Kaur and Halliwell, 1994), has been observed in liver and sinusoidal endothelial cells (Hinson et al., 1998; Knight et al., 2001; Knight and Jaeschke, 2004) of

AP treated mice. In addition, time course studies indicated that nitrotyrosine preceded or developed parallel to cell injury in both hepatocytes and endothelial cells (Knight et al., 2001). Peroxynitrite (PN) is formed by a reaction between nitric oxide (NO) and superoxide and some researchers have found increased NO synthesis during AP toxicity. (Hinson et al., 1998) In addition to nitrating tyrosine, PN is a pro-oxidant which can damage biomolecules (Pryor and Squadrito,1995). GPx is believed to be a key enzyme in detoxifying PN and due to GSH depletion its activity is reduced (Sies et al., 1997), leading to a decreased ability to deal with oxidative stress. However, GPx1 detoxification of PN is still debatable since some research has found that hepatocytes from GPx1-/- mice are resisitant to PN induced mortality (Fu et al., 2001).

Reports regarding whether PN formation is responsible for cellular injury are conflicting. Some have found partial protection against AP induced liver damage with inducible Nitric Oxide Synthase (iNOS) inhibitors and in iNOS knockout mice (Gardner et al., 1998; 2002). Others did not observe these protective effects (Michael et al., 2001; Hinson et al., 2002). Knight et al., (2001) suggested that PN formation occurred without iNOS induction, indicating that NO production by a constitutively expressed NOS produces sufficient NO for PN production.

GSH is able to detoxify PN in vitro and prevent nitrotyrosine formation (Kirsch et al., 2001; Knight et al., 2002). Decreased GSH following AP overdose may contribute to both protein nitration and PN toxicity (Knight et al., 2002).

Diquat and Paraquat, Inducers of Reactive Oxygen Species

Diquat dibromide (DQ; 1,1'-ethhylene-2,2'-dipyridylium dibromide) is an herbicide that has been associated with acute liver necrosis. The molecule is oxidized to a cation that reacts quickly with oxygen to reform the parent compound and a molecule of superoxide. This redox cycling continues as long as reducing equivalents are available and is able to produce large amounts of superoxide with only a small amount of DQ and unlike many toxicants it is not detoxified by alkylation or conjugation. Toxicity is due to oxidative stress (Daniel and Gage, 1966; Spalding et al., 1989) causing protein thiol oxidation and lipid peroxidation, inducing necrosis in vivo (Smith 1985; 1987a; 1987b). The liver is a primary target of these toxic effects (Burk et al., 1995).

Paraquat (PQ or methyl viologen; 1'-dimethyl-4,4'-bipyridylium dichloride) is a compound with biochemical properties similar to those of DQ. It is a widely used herbicide that is toxic to both plants and animals (Bus, 1976). At high doses most PQ is accumulated in the lung and eliminated by the kidney (Smith, 1987c). Due to structural similarities, preferential transport to the lung is believed to be via an endogenous polyamine pathway into type I and type II alveolar epithelial cells (Gordonsmith et al., 1983). In humans, a non-toxic dose is 90% eliminated into urine by the kidney within12 – 24 h (Bismuth et al., 1987). In the case of a toxic dose, the renal tubule cation transport system eliminates PQ (Groves et al., 1995). Similar to DQ, PQ is reduced to a radical form by a NADPH dependent pathway. The PQ radical then reacts with molecular oxygen to form a PQ cation and superoxide anion. By being reduced to a radical again, PQ can repeatedly cycle and produce large amounts of superoxide. Cagen and Gibson (1977) showed that Se deficient mice were sensitive to PQ toxicity and Cheng et al., (1998) showed in a GPx1

knockout mouse model that GPx1 was the mediator of selenium's protection against acute paraquat induced oxidative stress.

Plasma Alanine Aminotransferase and Protein Carbonyl

Plasma alanine aminotransferase (ALT) is a frequently used in clinical settings to assess liver function. Activity of ALT can increase rapidly in the plasma in the presence of xenobiotics that cause liver necrosis (e.g. AP and DQ) (Daniel and Gage, 1966; Prescott, 1980; Flanagan et al., 1995). ALT stored in hepatocytes is released when hepatocytes are acutely damaged. Increases in plasma concentrations of this enzyme provide important evidence of hepatocyte damage (Rosenthal et al., 1997).

Protein carbonyl is commonly used as a marker of generalized oxidative damage within the cell. Since the addition of carbonyl groups to proteins by oxidative modification occurs by many mechanisms, it can provide useful information about many oxidative processes. The accumulation of oxidized protein is dependent on many factors including: rates of ROS formation, antioxidant levels, and the ability to proteolyze and eliminate oxidized proteins (For review, see: Stadtman and Levine, 2003).

Unanswered Research Questions

Previously, whole animal work on GPx4 was limited to Se deficient models which could not specifically address GPx4 without involvement of other selenoproteins. Since it is resistant to Se depletion and a full knockout is embryonic lethal, this protein apparently has necessary functions. Many questions have not been addressed in whole animal models due to the limitations of older models. Since this half-knockout model provides the first opportunity to independently test effects of reduced GPx4, we have used

these mice to address the following questions in Experiment 1: Are there changes in the expression of GPx4 in various tissues of haploid insufficient mice? Does altered GPx4 expression have an effect on tissue selenium concentration or the expression of other selenoperoxidases? Does GPx4 have an antioxidant function that is compromised by deletion of one *gpx4* gene? Given the important functions for reproduction and high expression of GPx4 in the testis, are there any apparent changes in the fertility of male mice?

Oxidative (involving ROS) and nitrosative (involving RNS) processes are involved in many processes in the cell, some physiologically normal and others which can disrupt normal cell function. Reactive oxygen species are a by-product of aerobic metabolism which can cause cellular damage. It has been assumed that loss of function of antioxidant enzymes (e.g. GPx1 or SOD1) is detrimental under nearly all conditions. However, previous work in our lab has shown that this is not always the case since GPx1 knockout decreased hepatocyte sensitivity to peroxynitrite (Fu et al., 2001) and increased hepatocyte and whole animal ROS sensitivity. In Experiment 2 we address the following questions: Does this opposite relationship of ROS/RNS sensitivity also hold true in the whole animal? Does knockout of a different antioxidant enzyme (SOD1) have a similar effect? How does partial or complete loss of SOD1 and GPx1 genes in combination affect these responses? How does partial or complete loss of SOD1 and GPx1 genes in combination affect enzyme activities?

Objectives:

Experiment 1

To examine the effect of deletion of a single copy of gpx4 on:

1. The activities of three selenoperoxidases (GPx1, GPx3, and GPx4) in lung, liver, kidney and testis
2. Selenium concentrations in lung, liver, kidney and testis
3. Susceptibility to pro-oxidant-induced protein oxidation in lung, liver, kidney and testis
4. Fertility in male mice

Experiment 2

To use mouse models carrying mixed SOD1 and GPx1 knockouts (possessing 0, 1 or 2 functional copies of each gene in various combinations) to:

1. Determine effect of various combinations of GPx1 and SOD1 gene dosage on GPx1 and SOD activity
2. Determine effect of GPx1 and SOD1 gene dosage separately and in combination on mouse susceptibility to diquat and acetaminophen toxicity and lethality
3. Examine effect of diquat and acetaminophen toxicity on ALT, GPx1 and SOD enzyme activity

CHAPTER 2:

MATERIALS AND METHODS

All chemicals and materials were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Mice

Animal care

Mice were bred and housed in the facilities of the Department of Animal Science at Cornell University. All mice were housed in standard "shoebox" style cages (n=1-4 per cage) on stainless steel racks at 70-72°F and 35-55% humidity with12 h light/12 h dark cycle. All mice were given distilled water *ad libitum*. Mice from Experiment 1 were fed a vitamin E and selenium adequate diet (90.18 IU/kg and 0.33 mg/kg, respectively) (rodent diet #8604, Harlan-Teklad Madison, WI) *ad libitum*. In Experiment 2, mice were fed 5 g/d of a torula yeast based Se adequate diet previously described by Cheng et al., (1997). All studies were approved by the Institutional Animal Care and Use Committee at Cornell University and conducted in accordance with NIH guidelines for animal care.

Experiment 1. GPx4 Knockout Mice

GPx4+/- mice (generation described by Yant et al., 2003) were provided by Dr. Tomas Prolla of the University of Wisconsin at Madison. As GPx4 nullizygous is embryonic lethal, all mice born were either GPx4+/- or WT, providing an internal control. Mice were genotyped by a PCR method. Primers for detection of GPx4 wild-type and knockout alleles are:

Forward primer: 5’-GGCCTGGTTTCTATGTA-3’

Wild-type reverse primer: 5’-CCCCTGCCCTTCTGGACTATTGG-3’

Knockout reverse primer: 5’-GCACACTGGCAAAACAATGC-3’ .

Experiment 2. GPx1 and SOD1 Knockout Mice

Generation and characterization of GPx1 and SOD1 knockout mice was previously described by Ho et al., (1997, 1998). Both strains of mice were interbred and produced offspring carrying 0, 1 or 2 functional copies of each gene. Mice were genotyped by PCR. Primers for detection of GPx1 wild-type and knockout alleles are:

Forward primer: 5'-GTTTCCCGTGCAATCAGTTCG-3'

Wild type reverse primer: 5'-TCGGACGTACCCTTGAGGGAAT-3'

Knockout reverse primer: 5'-CATTTGTCACGTCCTGCAC-3'

Primers for detection of SOD1 wild-type and knockout alleles are:

Forward primer: 5’-GGACATCGTGTGATCTCACTCTCAGGAGAG-3’

Wild type reverse primer: 5’-CAAGCGGCTCCCAGCATTTCCAGTCTTTGT-3’

Knockout reverse primer: 5’-AAAAGCGCCTCCCCTACCCGGTAGAATTGA-3’

Treatments

Experiment 1. Paraquat: Mice were given IP injections with 24 mg/kg body weight paraquat (methyl viologen, PQ) as 2.4 mg PQ/ml in phosphate buffered saline, 0.2 micron sterile filtered or an equivalent volume of sterile filtered phosphate-buffered saline (PBS). Mice were euthanized 4 h post-injection by carbon dioxide asphyxiation followed by exsanguination with a heparinized syringe.

Experiment 2. Diquat and Acetaminophen: Mice were given intraperitoneal (IP) injections with 24mg/kg body weight diquat (diquat dibromide, DQ) as 2.4 mg DQ/ml in phosphate bufered saline (PBS) or 600 mg/kg body weight acetaminophen (paracetamol, AP) as 60 mg AP/ml in PBS or an equivalent volume of PBS. All solutions were 0.2 micron sterile filtered prior to injection. Mice were monitored until death, for up to 72 hours. Tissue and plasma samples from mice that died were collected immediately after death, mice which survived to 72 hours were euthanized by carbon dioxide asphyxiation followed by exsanguination with a heparinized syringe.

Sample Collection and Preparation

Experiment 1. Two plasma samples were taken from the blood post-exsanguination. One was used fresh for plasma alanine aminotransferase activity assay and one was snap frozen in liquid nitrogen for later GPx3 analysis. Lung, liver, kidney and testes were collected, rinsed in 0.9% saline and snap frozen in liquid nitrogen. All frozen samples were stored at -80°C.

Experiment 2. A fresh plasma sample was used for plasma alanine aminotransferase activity (ALT) assay. Liver was collected, rinsed in 0.9% saline, snap frozen in liquid nitrogen and stored at -80° C.

Enzyme assays

Plasma alanine aminotransferase activity (plasma ALT) was assayed using the Infinity ALT reagent (Thermo Electron Corp. Waltham, MA) at 30°C, according to manufacturer's instructions. Tissue homogenates were prepared as described in Cheng et al., (1997). In brief: 1:20 dilutions (w/v) of tissue in homogenization buffer (0.25 mol/L sucrose containing 20 mmol/L Tris-HCl, pH

7.4 for GPx1 and GPx3, 0.25 mol/L sucrose containing 20 mmol/L Tris-HCl, pH 7.4, 0.1% triton, for GPx4) were homogenized on ice and centrifuged at 105,000g for 1 hour. Supernatants were collected and frozen at –80°C. Activities of GPX1 and GPX4 in tissue homogenates and plasma were measured by the NADPH coupled assay, using hydrogen peroxide and phosphatidylcholine hydroperoxide as substrate, respectively (Cheng et al., 1997). Protein was measured by the Lowry method (Lowry et al., 1951) and enzyme activity expressed as Units/mg protein. Superoxide Dismutase activity was measured in GPx1/SOD1 liver using the SOD Assay Kit - WST from Dojindo (Gaithersburg, MD) according to manufacturer's instructions.

Tissue Selenium Concentration

Tissue selenium concentration was determined on whole tissue samples using the improved fluorometric method of Olson et al., (1975) and expressed as μg/g wet tissue weight.

Protein Carbonyl

As a hallmark of protein oxidation, total protein carbonyl was determined by a spectrophotometric method based on Levine et al., (1994) and Reznick and Packer (1994) and expressed as nmol/mg protein.

Statistics

Data were analyzed using SAS (release 9.1, SAS Institute, Cary, NC). Significance was defined as $P < 0.05$ and results are expressed as mean **±** SEM.

Experiment 1. Data were analyzed using the GLM procedure in SAS as a 2 x 2 factorial ANOVA. Tukey's test was used for mean comparisons.

Experiment 2. Treatment survival was modeled in two ways using SAS. Both a general linear model (GLM procedure) and survival analysis model (TPHREG procedure) were used to compare the effects of genotype and treatment. For the general linear model, the outcome measure *survival time* was assigned four classes (1 = <20 h, 2 = >20 - 40 h, 3=>40 h - <72 h, 4=survived experiment, 72+ h) and was used with genotype and treatment as predictors in a multi-factorial ANOVA to determine main effects. Scheffe's test was used for mean comparisons. The TPHREG procedure is based on the Cox proportional hazards regression and produces a description of a time dependent survival curve. Genotypes were classified according to copy number of either WT or KO genes [e.g. -/+ = 1(-) or 1(+) and -/- = 2(-) or 0(+)]. To separate effects of each gene (GPx1 or SOD1), analyses were stratified for the copy number of the other gene (SOD1 or GPx1). Models using all factors were also employed to determine effects in each experimental group. Hours of survival was used as the outcome measure (Allison, 1995; Hosmer and Lemeshow, 1999).

Enzyme values were also analyzed by the GLM procedure, using the same survival time classification described above. Enzyme values were then grouped by and analyzed as a multi-factorial ANOVA with survival class, GPx1 genotype, SOD1 genotype and treatment as predictors with Tukey's test for mean comparisons. For all statistical analyses, significance was defined as $P<0.05$.

CHAPTER 3:

EFFECTS OF GPX4 HAPLOID INSUFFICIENCY ON GPX4 ACTIVITY, SELENIUM CONCENTRATION, AND PARAQUAT-INDUCED PROTEIN OXIDATION IN VARIOUS TISSUES OF MICE

Abstract

Selenium-dependent glutathione peroxidase-4 (GPx4) catalyzes the reduction of phospholipid hydroperoxides. Since a full *gpx4* knockout is embryonic lethal, we examined the effect of deletion of one copy of *gpx4* on the activities of three selenoperoxidases (GPx1, GPx3, and GPx4), selenium concentrations, and pro-oxidant-induced protein oxidation in various tissues of mice. A total of 32 GPx4 hemizygous (GPx4+/-) and wild-type (WT) mice (8-10 weeks old, 16 males and 16 females) were fed a selenium-adequate diet and given an intraperitoneal injection of paraquat (PQ, 24 mg/kg of body weight) or phosphate buffered saline (PBS). All mice were euthanized 4 h after injection to collect tissues for analyses. In PBS-treated mice, GPx4 activities in lung, liver, kidney, and testes of GPx4+/- mice were 24-39% lower ($P < 0.05$) than in WT. Among PQ-treated mice, only testes GPx4 activity in GPx4+/- mice was significantly lower (54%, $P < 0.05$) than WT. Selenium concentration in testes, but not in other tissues, was reduced (34%, $P < 0.05$) in GPx4+/- mice compared with WT, irrespective of treatment. Tissue GPx1 activities and plasma GPx3 and alanine aminotransferase activities were unaffected by PQ treatment or *gpx4* hemizygosity. Total protein carbonyl was elevated (73%, $P < 0.05$) by PQ only in lung, and this effect of PQ was independent of genotypes. In conclusion, *gpx4* haploid insufficiency reduced GPx4 activities

and(or) selenium concentrations, but had no effect on pro-oxidant-induced protein oxidation in various tissues of mice.

Introduction

Phospholipid hydroperoxide glutathione peroxidase or GPx4 was discovered by Ursini et al., (1982) as a new selenoprotein from pig liver extract with the ability to protect cellular lipids against peroxidation and to reduce phosphatidylcholine hydroperoxides. Being distinct from cellular glutathione peroxidase-1 (GPx1) (Schuckelt et al.; 1992), GPx4 is expressed as three isoforms with alternate start codons and exons: a 23 kDa form (with a 27 amino acid mitochondrial targeting sequence that is later cleaved), a 20 kDa non-mitochondrial form and a 34 kDa sperm nucleus form (with an alternate first exon) (Pushpa-Rekha et al., 1995; Arai et al., 1996). Compared with other seleno-GPx proteins, GPx4 shares approximately 30 to 40 % nucleotide identity (Imai and Nakagawa, 2003). The enzyme functions as a monomer rather than a tetramer (as in the case of other GPx proteins), and it is the only GPx that is able to reduce phospholipid hydroperoxides (Ursini et al,. 1985). Nutritionally, GPx4 is much more resistant to dietary Se deficiency than the other GPx enzymes, particularly GPx1. When liver GPx1 activity and protein are reduced to nearly zero in selenium-depleted rodents (Weitzel et al., 1990; Lei et al., 1995; Bermano et al., 1996), liver GPx4 activity maintains approximately 20% of the selenium adequate levels (Weitzel et al., 1990; Thompson et al., 1998). However, the relative portion of total tissue Se in the form of GPx4 protein and the effect of GPx4 expression on the expression of other selenoperoxidases in various tissues are unclear, and could not be determined using conventional selenium-deficient animal models.

Yant et al., (2003) found that a full knockout of *gpx4* in mice is lethal at embryonic day 7.5 and postulated an essential function of GPx4 in development. It is well known that GPx4 protein and activity are very high in testes (Weitzel et al., 1990; Roveri et al., 1992). Ursini et al., (1999) reported that GPx4 is involved in sperm maturation and serves a structural role in the sperm tailpiece in an oxidatively cross-linked state, offering an explanation for the detrimental effects of Se deficiency on male reproductive function (Foresta et al., 2002). In addition, GPx4 has been proposed as a modulator of inflammatory lipid signaling molecules of various eicosanoids (Haurand and Flohe, 1988; Weitzel and Wendel, 1993; Schnurr et al., 1996; Imai et al., 1998; Huang et al.. 1999). However, GPx4 was initially suggested as an important antioxidant enzyme (Ursini et al., 1985). Although GPx4 haploid insufficiency was shown to render murine embryonic fibroblasts susceptible to pro-oxidant-induced oxidative stress (Yant et al., 2003; Ran et al., 2003*)* such *in vivo* impacts of GPx4 have not been well studied.

Since homozygous GPx4 knockout is lethal (Yant et al., 2003), we used the newly developed GPx4 hemizygous (GPX4+/-) mice (Yant et al., 2003) in the present study to examine the effect of deletion of a single copy of *gpx4* on the activities of three selenoperoxidases (GPx1, GPx3, and GPx4), selenium concentrations, and susceptibilities to pro-oxidant-induced protein oxidation in various tissues. Paraquat (methyl viologen, PQ) was chosen as the inducer of acute oxidative stress. This pro-oxidant compound primarily targets lung, a site of relatively high GPx4 activity (Weitzel et al., 1990; Bus et al., 1974; Smith, 1987). Since it is believed that PQ promotes the formation of superoxide radicals causing oxidative damage to important biomolecules, we chose

protein carbonyl formation as an indicator of protein oxidation (Levine et al., 1994).

Materials and Methods:

Mice. The generation of GPx4+/- mice was previously described (Yant et al., 2003). All mice were bred and housed in the mouse facility at Cornell University and were given free access to a selenium adequate (0.33 mg Se/kg) rodent diet (#8604, Harlan-Teklad Madison, WI) and distilled water. Genotypes of mice were verified by a PCR method modified from the one previously described (Yant et al., 2003), using a different reverse knockout primer located within the *hprt* cassette: 5'-gcacactggcaaaacaatgc-3'. Our study was approved by the Institutional Animal Care and Use Committee at Cornell University and conducted in accordance with NIH guides for animal care.

Experimental Procedure. A total of 32 GPx4+/- and WT mice (half male, half female, 8-10 weeks old) were assigned to treatments with PQ or phosphate-buffered saline (PBS) in a 2 x 2 factorial design (n = 6 to 10 per genotype by treatment). Mice were given an ip injection of 24 mg of PQ/kg of body weight (PQ was dissolved in PBS at 2.4 mg PQ/mL and passed through a 0.2 micron sterile filter) or an equivalent volume of sterile filtered PBS. All mice were euthanized at 4 h after injection by carbon dioxide asphyxiation followed by exsanguination with a heparinized syringe. The PQ dose and treatment interval were chosen based on responses of mice with the same genetic background to various doses of pro-oxidants and different lengths of exposure time (Cheng et al., 1998; 1999; 2003).

Two plasma samples were prepared from the blood: one was used fresh for plasma alanine aminotransferase activity assay and the other was snap frozen in liquid nitrogen for later GPx3 activity analysis. Lung, liver, kidney and testes were collected, rinsed in 0.9% saline and snap frozen in liquid nitrogen. All snap frozen samples were stored at -80°C before analysis.

Biochemical Analyses. All chemicals and materials were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Plasma alanine aminotransferase (ALT) activity was assayed using the Infinity ALT reagent (Thermo Electron Corp. Waltham, MA) at 30°C, according to manufacturer's instructions. Tissue homogenates were prepared as previously described (Cheng et al., 1997). Activities of GPx1 and GPx4 in tissue homogenates were measured by the NADPH coupled assay, using hydrogen peroxide and phosphatidylcholine hydroperoxide as the substrate, respectively (Cheng et al., 1997). GPx3 was measured in plasma using the same assay as GPx1. Protein was measured by the Lowry method (28). Tissue selenium concentration was determined using the improved fluorometric method of Olson et al., (1975) and expressed as g/g wet tissue weight. As a hallmark of protein oxidation, total protein carbonyl was determined by a spectrophotometric method based on Levine et al., (1994) and Reznick and Packer (1994) and expressed as nmol protein carbonyl/mg protein.

Statistics. Data were analyzed using the GLM procedure in SAS (release 9.1, SAS Institute, Cary, NC) as a 2 x 2 factorial ANOVA for main effects. Tukey's test was used for mean comparisons. Significance was defined as $P < 0.05$. Results are expressed as mean **±** SEM.

Results

All animals survived the PQ injection and were apparently healthy throughout the 4 h treatment. Upon euthanasia, no gross abnormalities of internal organs were observed.

Tissue GPx4 and GPx1 Activities. Testes GPx4 activity was 36% ($P < 0.05$) and 54% ($P < 0.01$) lower in the PBS and PQ-treated GPx4+/- mice than that of the WT mice, respectively (Figure 3.1). Paraquat treatment enhanced testes GPx4 activity in the WT mice ($P < 0.05$), but not in the GPx4+/- mice. Liver, lung, and kidney GPx4 activities in the PBS-treated GPx4+/- mice were 24, 27 and 39 % lower ($P < 0.05$) than the PBS-treated WT mice, respectively. The PQ treatment did not cause significant difference in GPx4 activities between the two genotypes in these three tissues. There was no PQ or genotype effect on GPx1 activities in any of these tissues (Table 3.1).

Plasma ALT and GPx3 Activities*.* The PQ injection caused no changes in plasma ALT or GPx3 activities in either genotype (Table 3.1). There were no genotype differences in plasma ALT or GPx3 activities within either treatment.

Tissue Selenium Concentrations*.* Testes selenium concentration in the GPx4+/- mice was 34% lower ($P < 0.01$) than in WT mice regardless of the treatment (Figure 3.2). There was no treatment or genotype effect on selenium concentrations of other tissues (Table 3.1).

Protein Carbonyl Concentrations*.* Total protein carbonyl in lung of the PQ-treated mice was 73% higher ($P < 0.05$) than in the PBS-treated mice, irrespective of genotypes (Figure 3.3). No significant effects of genotype or treatment were shown on total protein carbonyl in liver, kidney or testes.

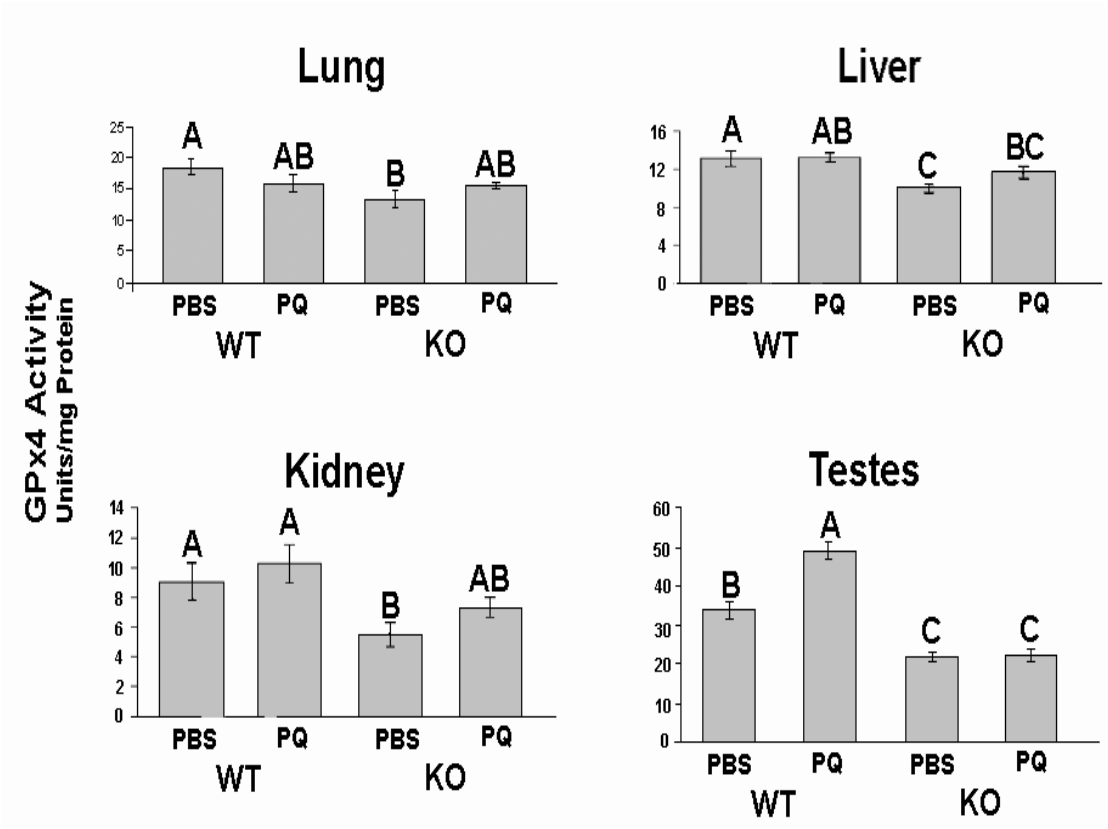

Figure 3.1. Effect of GPx4 Knockout and Paraquat Treatment on GPx4 Activity. PBS treated GPx4+/- mice showed 22%, 27%, 39% and 36% lower activity in the lung, liver, kidney and testes than WT, respectively ($P < 0.05$).Values are means ± SEM (n = 3 for testes, n = 6 for other tissues). Values with different letters are significantly different at $P < 0.05$.

Table 3.1. Effect of GPx4 hemizygosity and paraquat treatment on GPx1, GPx3 and ALT activity and tissue selenium concentrations

	WT		GPx4+/-	
	PBS	PQ	PBS	PQ
Cellular glutathione peroxidase (GPx1) (nmol GSH oxidized per min • mg protein)				
Lung	123.4 ± 10.1	127.4 ± 12.5	113.4 ± 11.4	131.8 ± 13.2
Liver	1042.0 ± 32.6	1157.4 ± 30.4	1078.5 ± 47.1	1114.9 ± 42.7
Kidney	733.1 ± 70.2	744.0 ± 25.7	803.6 ± 74.3	757.2 ± 35.3
Testes	76.0 ± 8.3	61.7 ± 8.8	86.3 ± 5.5	67.4 ± 8.6
Plasma glutathione peroxidase (GPx3) (nmol GSH oxidized per min • mg protein)				
	61.5 ± 4.1	71.8 ± 7.6	78.1 ± 9.3	59.4 ± 3.9
Plasma alanine aminotransferase (ALT) (Units/L)				
	99.5 ± 28.3	52.7 ± 19.6	82.7 ± 21.4	91.5 ± 34.3
Tissue selenium concentration (µg/g wet tissue weight)				
Lung	0.47±0.02	0.46±0.02	0.46±0.03	0.45±0.02
Liver	1.49±0.06	1.47±0.06	1.49±0.07	1.42±0.04
Kidney	1.34±0.05	1.35±0.06	1.33±0.06	1.25±0.04

No significant changes by genotype or treatment were found in lung, liver, kidney and testes GPx1 and plasma GPx3 activity, Plasma ALT activity and tissue selenium concentration of lung, liver and kidney. Values are means ± SEM, n = 6 for lung liver kidney and plasma, n = 3 for testes.

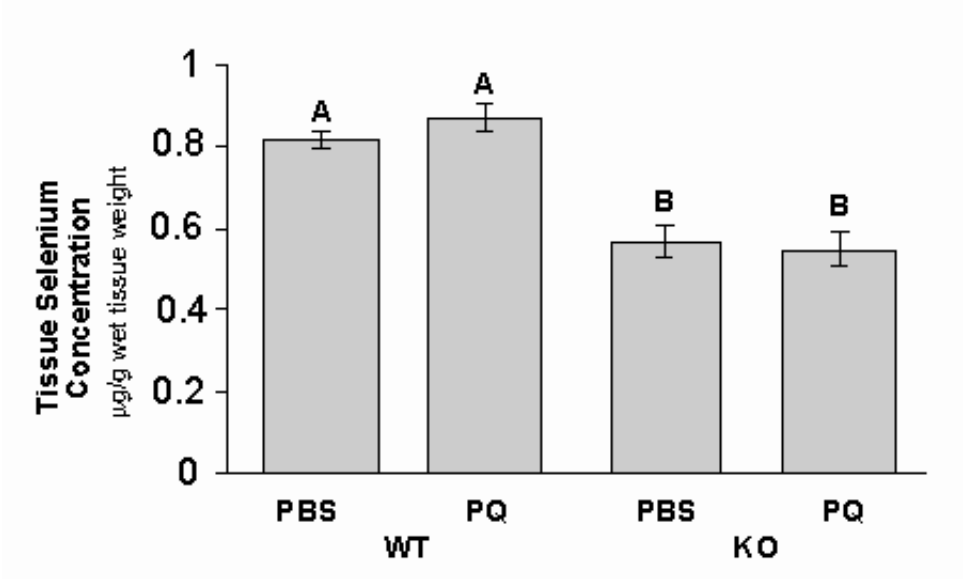

Figure 3.2. Effect of GPx4 Knockout and Paraquat Treatment on Testes Selenium Concentration. GPx4+/- testes exhibited a 31 – 37% lower selenium concentration than WT ($P < 0.05$). Values are means ± SEM ($n = 3$). Values with different letters are significantly different at $P < 0.05$.

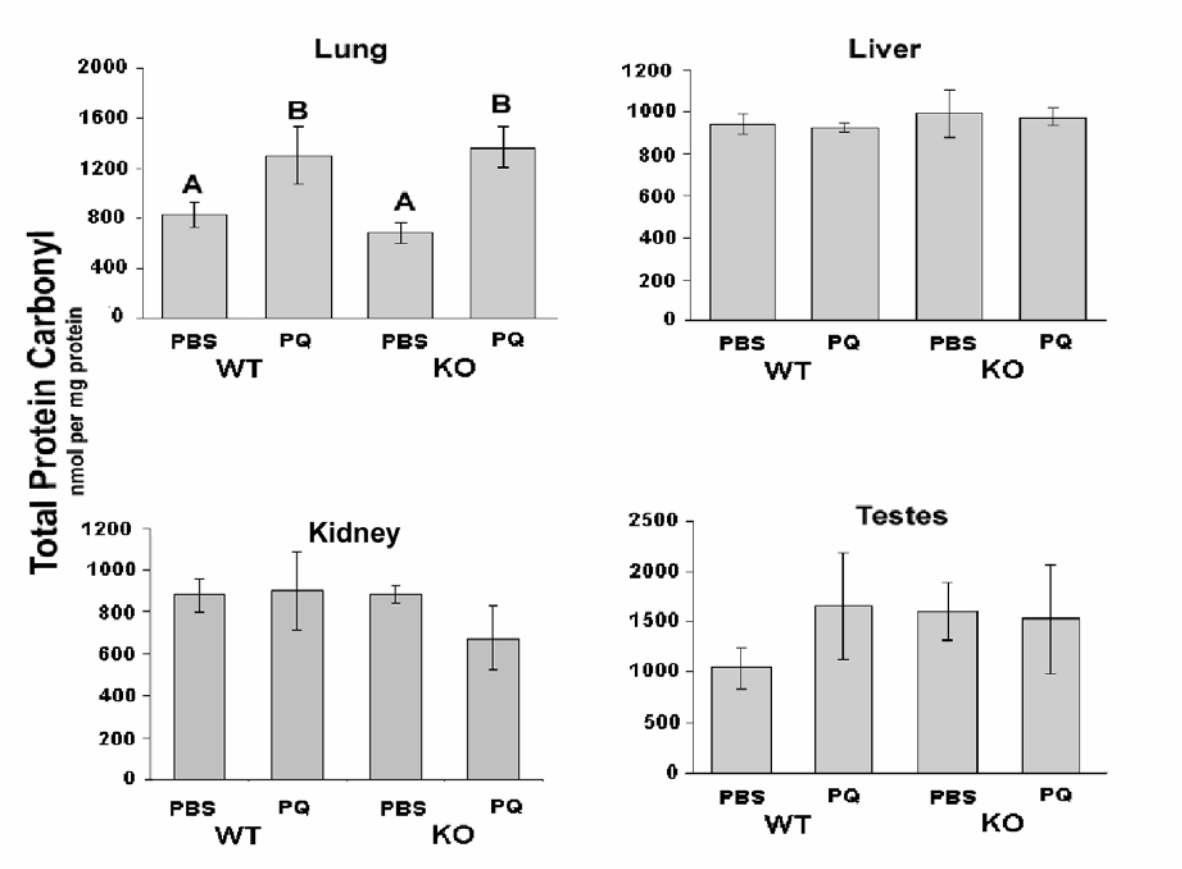

Figure 3.3. Effect of GPx4 Knockout and Paraquat Treatment on Total Tissue Protein Carbonyl. Overall, PQ treated mice had a 73% higher carbonyl concentration in the lung than PBS treated control ($P < 0.05$). Values are means ± SEM ($n = 3$ for testes, $n = 6$ - 10 for other tissues).Values with different letters are significantly different at $P < 0.05$.

Discussion

Our results provide us with three major novel findings. First of all, deletion of one copy of GPx4 resulted in a 24 to 39% baseline reduction in GPx4 activities in liver, lung, kidney, and testes, compared with the WT mice. Although Yant et al., (2003) and Ran et al., (2003; 2004) determined GPx4 mRNA and protein changes in various tissues and cells of GPx4+/- and GPx4 overexpressing mice, respectively, our study represents the first evidence for the effect of GPx4 haploid insufficiency on its enzymatic activity *in vivo*. Since changes in GPx4 mRNA or protein do not necessarily predict the actual activity of GPx4, it is necessary to know GPx4 activities in different tissues of GPx4+/- mice if the model is to be used for metabolic functional studies. For example, Yant et al., (2003) found an approximate 50% reduction in GPx4 mRNA and protein in the liver of GPx4+/- mice. However, we have found only a 24% reduction in GPx4 activity. Similarly, Yant et al., (2003) showed the lowest percentage reduction of GPx4 mRNA and protein in testes of GPx4+/- mice, whereas we have demonstrated that the GPx4 allelic deletion produced the highest percentage change in overall GPx4 activity (average 40% decrease in PBS and PQ treated groups) in the testes. Overall, the deletion of one allele of *gpx4* did not lead to 50% activity reduction in any tissue, contrary to some other cases of gene knockout (Huang et al., 1996; Kline et al., 2002; McIlvain et al., 2003). Thus, GPx4 activity or expression of the remaining allele of *gpx4* might be up-regulated somewhat in these tissues to compensate for the deletion.

Our second interesting finding that GPx4 haploid insufficiency resulted in the reduction of selenium concentration in testes, but not in other tissues. This implies that a relatively small amount of tissue selenium was associated

with the reduction of GPx4 activities in lung, liver and kidneys. In contrast, selenium concentration in testes of GPx4+/- was reduced in proportion to the activity reduction. Overall, the PBS and PQ-treated animals had an average of 40% reduction in GPx4 activity and a 34% reduction of testes selenium concentration. Accordingly, full expression of GPx4 should account for 85% of total selenium in testes. Since the PQ-associated GPx4 increase in activity in the testes of WT mice did not alter selenium concentration, post-translational regulation of the enzyme might be responsible for these changes under the present experimental conditions. A similar GPx4 response to oxidative stress has been reported in rat polymorphonuclear neutrophils in which GPx4 activity was up-regulated in response to increased oxidative stress during an inflammatory challenge (Hattori et al., 2005). Apparently, these up-regulations of GPx4 activity by pro-oxidant challenge depend on both genotype and tissue or cell type (Hattori et al., 2005). Since tissue GPx1 activities and plasma GPx3 activity were unaffected by GPx4+/-, the expression of these two selenoperoxidases is independent of that of GPx4. Our data extend a similar observation by Ran et al., (2003) in embryonic fibroblasts of GPx4+/- and are consistent with the impact of GPx4 overexpression on tissue GPX1 activity in mice (Ran et al., 2004). Conversely, Cheng and colleagues showed unchanged GPx3 and GPX4 activities in GPx1-/- mice (Cheng et al., 1998a).

Our third interesting finding is that reduction of GPx4 activities in liver, lung, kidney, and testes of GPx4+/- mice did not sensitize these tissues to the PQ-mediated protein-oxidation. This is contrary to the greater susceptibility of embryonic fibroblasts derived from GPx4+/- mice than those from wild-type mice to oxidative stress inducers of λ-irradiation, PQ, t-butyl hydroperoxide, hydrogen peroxide, and normoxic versus hypoxic conditions (Yant et al., 2003;

Ran et al., 2003). Obviously, results from cultured cells do not necessarily reflect the metabolic role of GPx4 under physiological conditions, and the level of oxidative stress employed in these cell studies may be higher than in the present study. Although the dose of PQ used in the present study did not affect survival or cause changes in plasma ALT activity and gross pathology, it did cause a significant increase in protein carbonyl formation in the target tissue of lung in both WT and GPx4+/- mice. This induction of protein oxidation in lung was comparable to that by other doses of PQ in liver of selenium-deficient mice in our previous studies (Cheng et al., 1998b; 1999) and in the lung of rat in studies by Winter et al., (1991). Thus, the 24 to 39% reduction in GPx4 activity in different tissues of GPx4+/- mice was not sufficient to compromise defense against pro-oxidant induced protein oxidation. As measurements were made at only one time point following only one level of PQ dosage in the present study, future research with various doses of PQ or other pro-oxidants targeting different tissues should be tested to further assess the role of GPx4 in *in vivo* anti-oxidation. Paraquat doses > 50 mg/kg body weight may produce sufficient oxidative stress to detect differences between WT and GPx4+/- mice (Levine et al., 1994). Diquat is structurally and functionally similar to paraquat and targets the liver rather than the lung. We have found doses of diquat in the range of 24 – 48 mg/kg to be useful for detecting changes in anti-oxidation (Fu et al., 1999b). Cadmium is believed to damage the testes by oxidative stress (Shen et al., 1995; Liu et al., 2001) therefore this heavy metal presents an opportunity to target an important site of GPx4 function with oxidative stress *in vivo*.

CHAPTER 4:

EFFECT OF GPX1 AND SOD1 GENE DOSAGE ON GPX1 AND SOD ACTIVITY AND SUSCEPTIBILITY TO ACETAMINOPHEN AND DIQUAT TOXICITY AND LETHALITY

Abstract

The objective of this study was to investigate the role of gene dosage of both GPx1 and SOD1 on acetaminophen (AP) and diquat (DQ) toxicity and the effect of these four factors on plasma ALT activity, liver GPx1 activity and liver SOD activity. Seventy-one adult mice were distributed to genotype and treatment groups, given intraperitoneal injections of 600 mg/kg body weight AP, 25 mg/kg body weight DQ or phosphate buffered saline (PBS) control and observed for 72 h. Plasma and liver samples were collected at time of death for enzyme assays. Overall, 26.7% of mice (n = 19) died during the 72 h experiment. DQ-treated SOD1-/- mice died at <20 h but survived the entire 72 h experiment with AP treatment. AP-treated SOD1+/+ mice showed a trend toward decreased survival but had full survival with DQ treatment. Similar general trends were found in both experimental and Cox Proportional Hazard (CPH) survival curves: SOD1-/- mice died rapidly from DQ but survived AP; SOD1+/+ mice survived DQ but died rapidly from AP; both AP or DQ treatment decreased survival of GPx1-/- mice but not GPx1+/+ mice; SOD1+/- and GPx1+/- mice had treatment responses intermediate between their respective +/+ and -/- genotypes. The effects of both genes were also considered: DQ-treated mice with a total of one functional GPx1 or SOD1 gene copy had significantly shorter survival than control (0-20 h versus >72 h, $P < 0.05$) but had no significant difference in survival when treated with AP.

AP-treated GPx1+/-|SOD1+/+ mice had significantly shorter survival time than control (20-40 h versus >40 h $P < 0.05$) and survived DQ treatment.

Plasma ALT activity response depended on survival time and treatment rather than directly on genotype. AP-treated mice which died in <20 h, at 20-40 h or 40 - <72 h showed significant ALT increases compared to PBS control, peaking at 20-40 h survival (3145 ± 877; 11740 ± 1074 and 6297 ± 1074 versus 152 ± 368 U/litre $P < 0.05$). DQ-treated mice who died in <20 h also had significantly increased ALT activity compared with control (2129 ± 537 versus 152 ± 368 U/litre $P < 0.05$). All surviving mice showed no significant increases in plasma ALT.

GPx1 genotype and SOD1 genotype had significant effects on liver GPx1 activity. All GPx1-/- and GPx1+/- mice showed significantly lower liver GPx1 activity than GPx1+/+ mice (1 ± 32 and 553 ± 30 versus 1014 ± 36 U/mg protein, $P < 0.05$). Among GPx1+/- mice, SOD1-/- had 34% lower liver GPx1 activity than SOD1+/- (364 ± 40 versus 553±30 U/mg protein, $P < 0.05$) and 28% lower activity than SOD1+/+ (364±40 versus 504±36 U/mg protein, $P = 0.0778$).

SOD1 genotype and survival time had significant effects on liver SOD activity. PBS treated SOD1-/- and SOD1+/- mice had significantly lower activity than SOD1+/+ mice ($P < 0.05$).

Although conclusions drawn from this study must be limited due to the sample size and low death rates in some groups, certain trends are seen. To summarize, the SOD1 knockout allele [SOD1(-)] is associated with decreased sensitivity to AP toxicity, increased sensitivity to DQ toxicity and decreased liver GPx1 activity and the GPx1 knockout allele [GPx1(-)] is associated with increased sensitivity to both AP and DQ toxicity.

Introduction

As the first identified mammalian selenoprotein, glutathione peroxidase 1 (EC: 1.11.1.9, GPx1) is a well characterized antioxidant protein (Flohe et al., 1972, Cheng et al., 1998b). GPx1 functions as a homotetramer of 88 kDa and each 23 kDa monomer contains one selenocysteine residue.

Although evidence in cell culture studies supported the role of GPx1 as an antioxidant enzyme (Mirault et al., 1991; Geiger et al., 1993), this function was first demonstrated in animals when Cheng et al., (1998b) used GPx1 knockout mice to show that GPx1 is the mediator of selenium's protection against acute oxidative stress. These findings were supported by the studies of others (De Haan et al., 1998) therefore there is now solid evidence for the role of GPx1 as a protector against acute oxidative stress. Interestingly, cell culture studies have shown that while GPx1 (Fu et al., 2001) protects against ROS produced by the superoxide generator diquat, it sensitizes hepatocytes to peroxynitrite induced apoptosis. The mechanism of the inverse relationship between sensitization to reactive oxygen species and reactive nitrogen species remains unexplained in cells and untested in animals.

Along with selenium, copper and zinc serve as cofactors in cytosolic antioxidant proteins. Copper, zinc superoxide dismutase (SOD1, EC 1.15.1.1), catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen which can then reduced to water by the action of GPx1 (see **Figure 1.1** and **Figure 1.2**) (McCord and Fridovich, 1969; Flohe et al., 1973; Rotruck et al., 1973). When superoxide production outpaces its elimination by SOD, superoxide can accumulate in the cell. Similarly, when hydrogen peroxide production outpaces GPx activity, hydrogen peroxide can accumulate. Under

these conditions, there is opportunity for these toxic metabolites to damage biomolecules.

To study the antioxidant role of SOD1, mice deficient in SOD1 (SOD1-/-) were generated and characterized (Ho et al., 1998). SOD1-/- mice were phenotypically normal and showed no change in sensitivity to hyperoxia (Ho et al., 1998). However, SOD1-/- mice did show increased sensitivity to acute paraquat (a ROS generator) toxicity and female mice demonstrated greatly reduced fertility compared to mice with one (SOD1 +/-) or two (SOD1+/+) functional copies of SOD1 (Ho et al., 1998). The mechanism for reduced female fertility remains unexplained.

The analgesic and antipyretic acetaminophen (AP) is considered very safe at normal doses but an overdose can produce a potentially fatal centrilobular hepatic necrosis (Prescott, 1980). When AP is metabolized, the cytochrome P450 system produces multiple metabolites, including *N*-acetyl-*p*-benzoquinoneimine (NAPQI). Normal detoxification of NAPQI is through conjugation with GSH to form an AP-GSH conjugate which is then eliminated (Mitchell et al., 1973). However, in the case of a toxic dose, total hepatic GSH is depleted and NAPQI can form AP-protein adducts (Mitchell et al., 1973). In addition to NAPQI, the superoxide anion is also formed and it is believed that ROS contributes to AP toxicity. In support of this, Nakae et al., (1990) reported that administration of encapsulated superoxide dismutase decreased the toxicity of acetaminophen in the rat.

Reactive nitrogen species (RNS) may also play a role in AP toxicity. Nitrotyrosine, an excellent biomarker of peroxynitrite formation (Kaur and Halliwell, 1994), has been observed in AP treated mice (Hinson et al., 1998; Knight et al., 2001; Knight and Jaeschke, 2004). In addition, timecourse studies indicated that nitrotyrosine preceded or developed parallel to cellular

injury (Knight et al., 2001). Peroxynitrite (PN) is formed by a reaction between nitric oxide (NO) and superoxide. In addition to superoxide production some researchers have found increased NO synthesis during AP toxicity (Hinson et al., 1998), providing both components of PN formation. In addition to nitration of tyrosine residues, PN is a pro-oxidant which can damage biomolecules (Pryor and Squadrito,1995). GPx is believed to be a key enzyme in detoxifying PN and due to the GSH depletion during of AP toxicity, its activity is reduced (Pryor and Squadrito,1995) leading to a decreased resistance to oxidative stress. The role of RNS in AP toxicity is still debatable. While some have reported partial protection against AP induced liver damage with inducible Nitric Oxide Synthase (iNOS) inhibitors and in iNOS knockout mice (Gardner et al., 1998; 2002), others did not observe these protective effects (Michael et al., 2001; Hinson et al., 2002).

The report of Fu et al., (2001) of GPx1 knockout protection against PN induced cell death in murine hepatocytes needs further *in vivo* investigation. Unfortunately, PN is too reactive to be used directly in animals. The putative role of PN in AP toxicity provides a way to increase RNS in an animal. In addition, the role of superoxide production in AP toxicity gives a further dimension to the question involving ROS, since mice lacking functional GPx1 or SOD1 have greatly increased ROS sensitivity (Cheng et al., 1998b; Ho et al., 1998). Given the importance of SOD1 and GPx1 in protecting against ROS and the resistance of GPx1 knockout cells to PN, how might the deletion of another antioxidant protein (SOD1) affect these responses? In this study, we examined the effects of various gene dosages of GPx1 and SOD1 in mice on diquat toxicity (an *in vivo* ROS generator) and AP toxicity (as a putative *in vivo* RNS producer).

Protocols

A total of 71 mice with various copy numbers of GPx1 and SOD1 genes were treated with intraperitoneal injections of 25 mg/kg body weight diquat (DQ), 600 mg/kg body weight acetaminophen (AP) or phosphate buffered saline (PBS) as control (see Table 4.1 for experimental group descriptions). Mice were fasted for 8 h before injection and given free access to food and water after injection and throughout the trial., Wild-type mice (GPx1+/+|SOD1+/+ [n = 6]) were included only in survival analysis and excluded from biochemical analyses. Mice were observed for 72 h post-injection and upon death, samples of liver and plasma were collected for GPx1, SOD and ALT activity assays. Time of death was recorded for all mice. All surviving mice were sacrificed at 72 h post-injection and the same samples collected. Plasma samples (from n = 69 mice) were used fresh for ALT activity assay. Liver samples (from n = 61 mice) were snap frozen in liquid nitrogen and stored at -80°C until homogenized for GPx1 and SOD activity assays.

Survival was analyzed using a general linear model for main effects and Scheffe's test for mean comparisons. Further survival analysis compared models from Cox proportional hazards analysis. Enzyme assays were analyzed using a general linear model for main effects and Tukey's test for mean comparisons. See Materials and Methods (Chapter 2) for a more detailed description of statistical analyses.

Table 4.1. Experimental groups in 72 h survival trial by GPx1 genotype, SOD1 genotype and treatment.

SOD1 Genotype		GPx 1 Genotype		
		+/+	+/-	-/-
+/+	AP	-	4	-
	DQ	-	4	-
	PBS	7	3	-
+/-	AP	4	6	6
	DQ	4	6	6
	PBS	3	4	4
-/-	AP	-	3	-
	DQ	-	4	-
	PBS	-	3	-

AP = 600 mg acetaminophen /kg body weight; **DQ** = 25 mg diquat /kg body weight; **PBS** = phosphate buffered saline; **+** = wild type allele; **-** = knockout allele

Results

All mice treated with PBS survived. Overall, 26.7% (n = 19 out of n = 71) mice from 5 of the 16 groups died within the 72 h observation period. Samples were not immediately collected from two mice which died and have therefore been excluded from all biochemical analyses but were included in survival analyses.

Survival: SOD1 Effects

AP-treated SOD1+/+ mice had a significantly lower survival time than SOD1+/- or SOD1-/- mice when compared within a general linear model (<20 h vs. >40 h and >72 h $P < 0.05$). DQ treatment showed the opposite effect: SOD1 -/- mice died within 20 hours but SOD1+/+ survived the entire 72 h observation period. When compared between treatments, SOD1-/- mice survived AP treatment but died in <20 h with DQ treatment and although the difference is non-significant in the general linear model, SOD1+/+ mice showed decreased survival when treated with AP and full survival with DQ treatment (Figure 4.1). Although the data could not be adjusted for the effect of the GPx1 genotype due to limitations of the size of the study, SOD1 genotype*Treatment survival curves of the experimental data show that DQ-treated SOD1 -/- mice had a rapid decline in survival while all DQ-treated SOD1+/+ mice survived the entire trial and SOD1+/- had survival rates intermediate between the two. The experimental data survival curves also show that with AP treatment SOD1+/+ had the greatest decline in survival, all SOD1-/- survived and SOD1+/- had survival rates between the two (Figure 4.2).

Effects of SOD1 genotype independent of GPx1 genotype were analyzed with Cox Proportional Hazards (CPH) models stratified for GPx1. A

model for AP survival time which showed significant correlation with experimental AP effect on SOD1 genotypes was based on a full description of the SOD1 genotype [SOD1(-) copy number*SOD1(+) copy number*AP], stratified for a full description of the GPx1 genotype [GPx1(-) copy number*GPx1(+) copy number]. In this model, SOD1-/- mice had no AP-induced mortality and mice possessing one or both functional copies of SOD1 (SOD1+/- or SOD1+/+) had decreased survival rates (68% and 19% survived to 72 h, respectively) (Figure 4.3).

A CPH model with significant correlation to the experimental data for the response of SOD1 genotypes to DQ treatment is based on SOD1(-) copy number*DQ stratified by either GPx1(-)copy number. This model shows that absence of one or both copies of SOD1 (SOD1+/- or SOD1-/-) is associated with a precipitous decrease in survival rate (100% of SOD-/- and >50% of SOD+/- died before 20 h while SOD1+/+ mice survived the entire DQ trial (Figure 4.3).

Survival: GPx1 Effects

When compared within general linear models, only DQ-treated GPx1-/- mice had a significantly shorter survival time (<20 h) than any other GPx1 genotype*Treatment groups ($P < 0.05$, Figure 4.1). Due to small sample size, these general linear models could not be adjusted for effects of SOD1 genotype.

Examination of GPx1 genotype*Treatment experimental data survival curves show that with either AP or DQ treatment GPx1-/- mice had the most rapid decline of survival, all GPx1+/+ mice survived and GPx1+/- mice had survival rates intermediate between the two. As noted above, these data were not

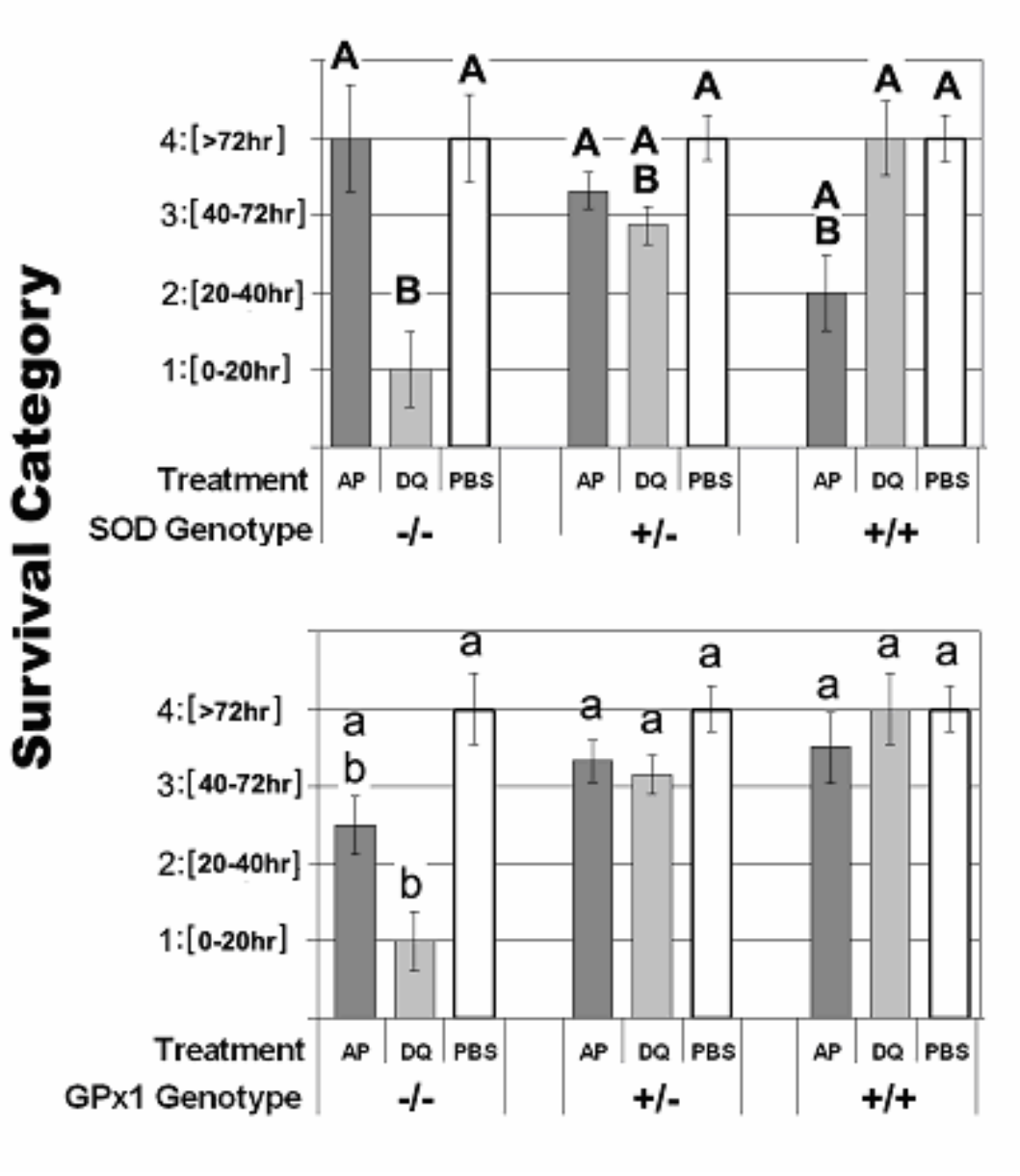

Figure 4.1. Effect of GPx1 or SOD1 genotype in mice on 72 h survival of acetaminophen or diquat treatment. Diquat treated SOD1-/- and GPx1-/- mice had significantly shorter survival than other mice. Although there were no significant differences, SOD1+/+ and GPx1-/- mice show a trend towards decreased survival., Values are means ± SEM (n = 3-16). Bars with different letters are significantly different at $P < 0.05$.

adjusted for the effect of the other genotype due to the limitations of study size (Figure 4.2).

In order to separate effects of GPx1 genotype from SOD1 genotype, data were analyzed using CPH models stratified for SOD1. These models are based on interaction of GPx1(+) copy number with treatment stratified by SOD1(+) copy number and show significant correlation with the experimental data survival curves ($P < 0.05$). In agreement with the experimental data, these models show that both AP- and DQ-treated GPx1-/- and GPx1+/- have decreased survival rates when compared with GPx1+/+ (Figure 4.4).

Survival: GPx1 – SOD1 Interactions

In the time categorized general linear model, mice with only one functional copy in total of both GPx1 and SOD1 (i.e. GPx1+/-|SOD1-/- or GPx1-/-|SOD1+/-) had significantly shorter survival time when treated with DQ than mice carrying more than one functional copy in total or PBS control (0-20 h versus >20 h, $P < 0.05$). Survival of mice with these same genotypes (GPx1+/-|SOD1-/- and GPx1-/-|SOD1+/-) treated with AP was not significantly different from control (>72 h). In comparison with PBS control, GPx1+/-|SOD1+/+ mice had significantly shorter survival time when treated with AP but had no change with DQ treatment (<20 h versus >72 h, $P < 0.05$). Mice with one functional copy of each gene (GPx1+/-|SOD1+/-) showed no difference in survival from control (>72 h versus >72 h) when treated with either DQ or AP (Table 4.2). From the survival curves of the experimental data, it can be seen that mice with the smallest number of functional copies of GPx1 and SOD1 (i.e. GPx1-/-|SOD1+/- and GPx1+/-|SOD1-/-) treated with DQ

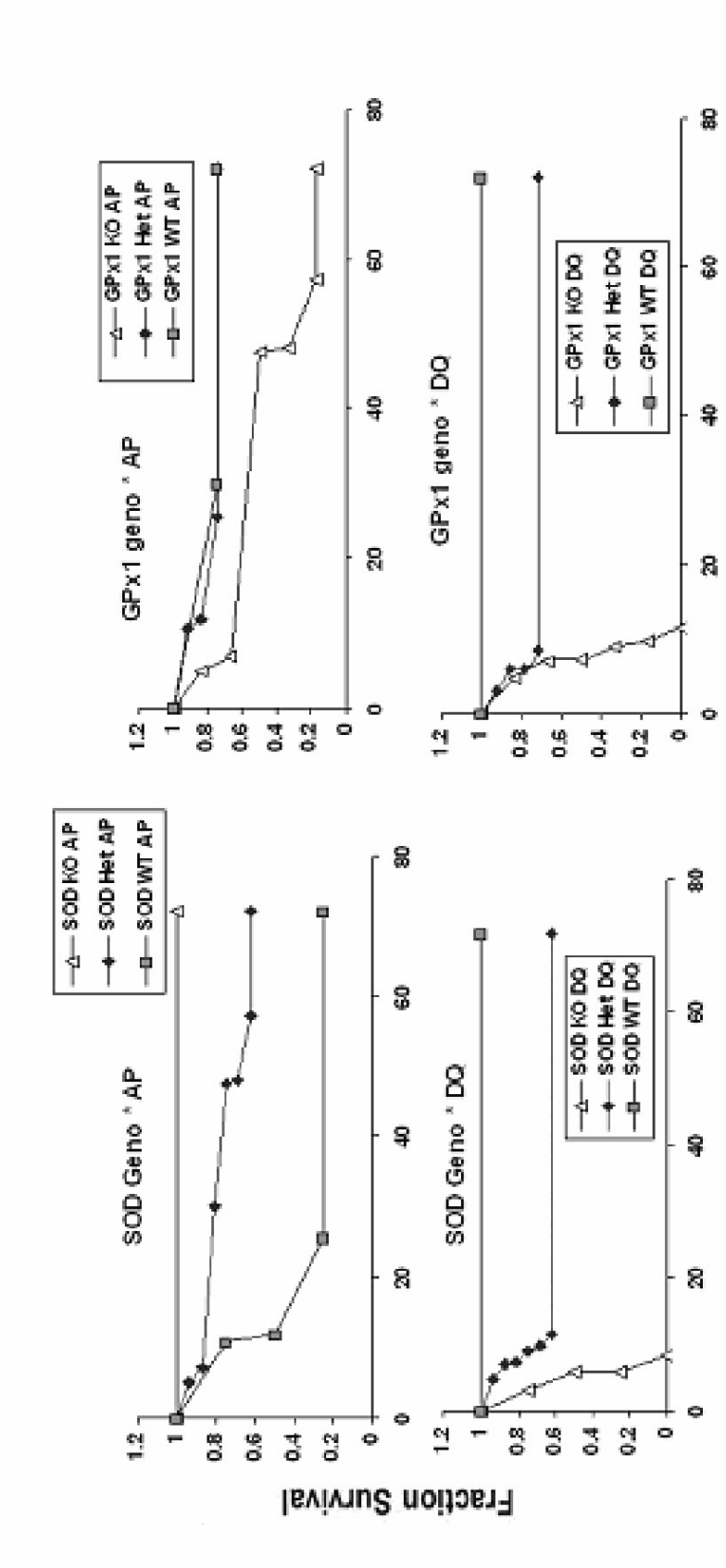

Figure 4.2. Seventy-two hour survival curves of acetaminophen or diquat treatment in mice with various gene dosages of GPx1 or SOD1. SOD1-/- and GPx1-/- mice die rapidly with diquat treatment while GPx1+/+ and SOD1+/+ survive. SOD1-/- mice survive acetaminophen treatment while SOD+/+ and GPx1-/- have decreased survival.

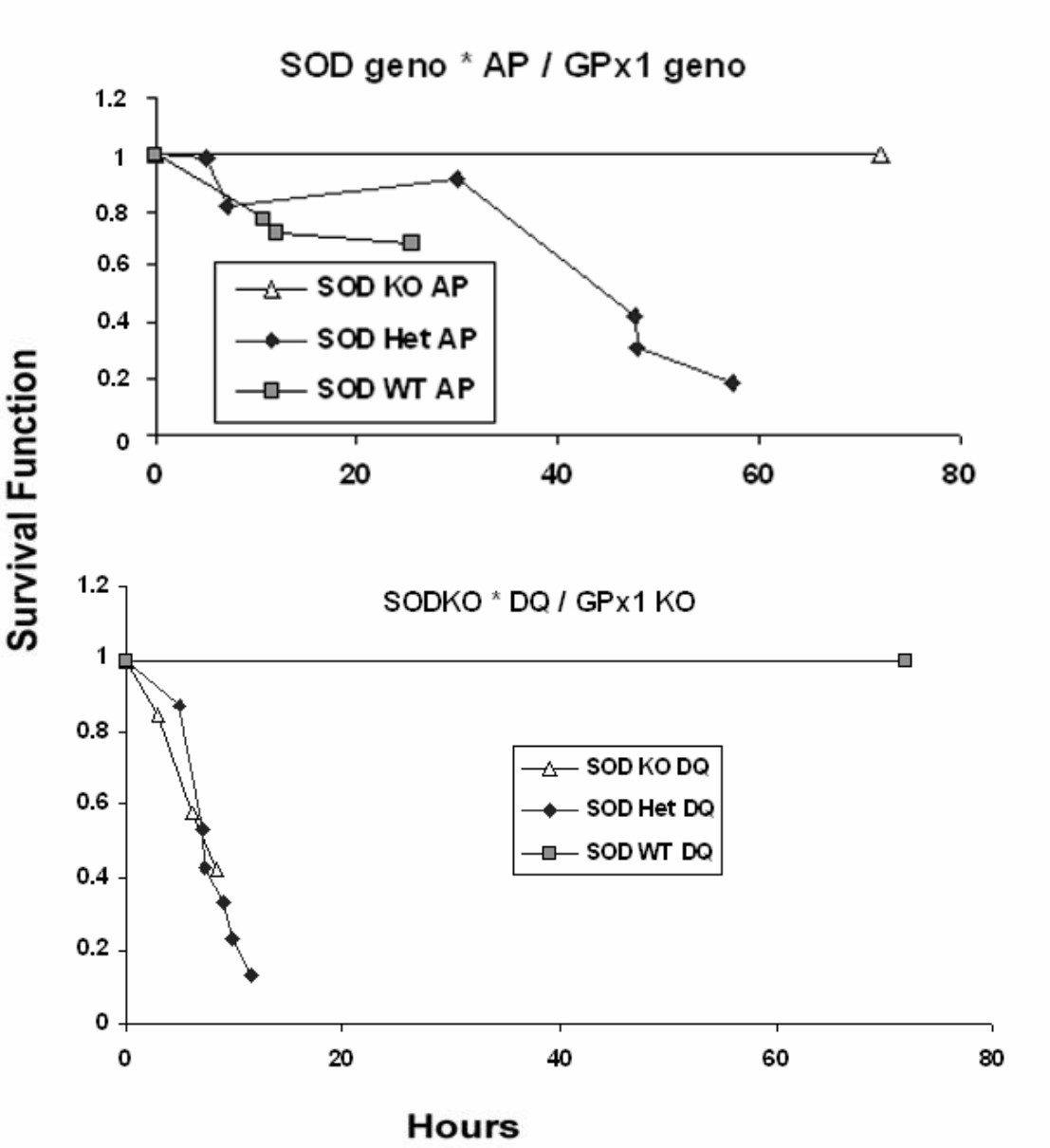

Figure 4.3. Seventy-two hour survival curves from Cox proportional hazards models of mice with various gene dosages of GPx1 and SOD1 treated with acetaminophen or diquat: SOD1*treatment effects. A Cox proportional hazards model showing high correlation ($P < 0.05$) with experimental SOD1 genotype response to acetaminophen is based on a full description of the SOD1 genotype stratified for a full description of the GPx1 genotype. The AP model shows that SOD1-/- mice survive acetaminophen treatment while SOD1+/+ mice die rapidly. A Cox proportional hazards model showing high correlation ($P < 0.05$) with experimental SOD1 genotype response to Diquat is based on SOD1(-) copy number stratified for GPx1(+) copy number. The DQ model shows that SOD1-/- mice die rapidly with diquat treatment and SOD1+/+ mice survive. **KO** = -/- , **Het** = +/-, **WT** = +/+.

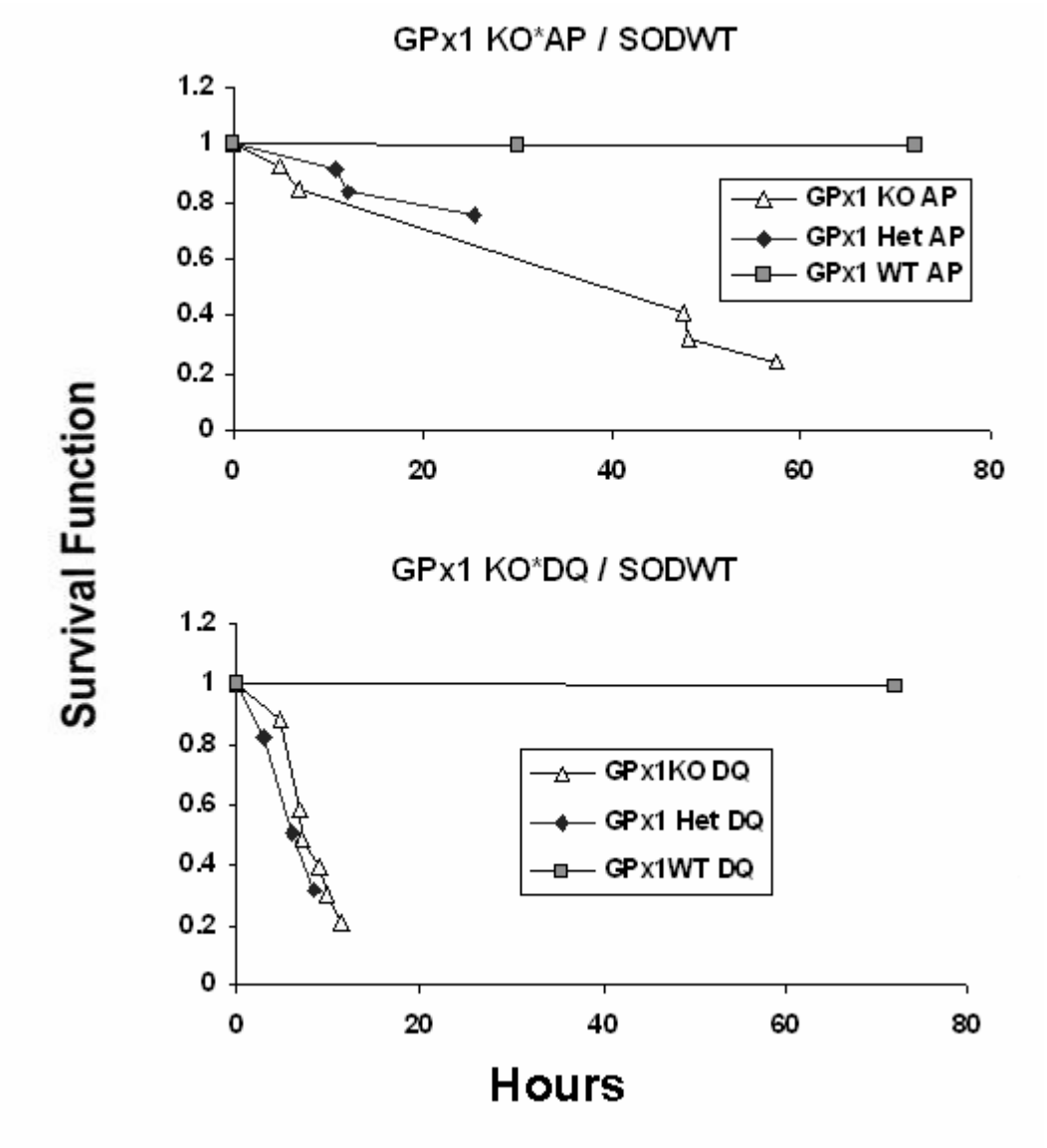

Figure 4.4. Seventy-two hour survival curves from Cox proportional hazards models of mice with various gene dosages of GPx1 and SOD1 treated with acetaminophen or diquat: GPx1*treatment effects.A Cox proportional hazards models showing high correlation ($P < 0.05$) with experimental GPx1 genotype response to acetaminophen or diquat is based on GPx1(-) copy number stratified for SOD1(+) copy number. The AP model shows that GPx1-/- mice have decreased survival with acetaminophen treatment while GPx1+/+ mice survive. Similarly, the DQ model shows that GPx1-/- mice die rapidly with diquat treatment and SOD1+/+ mice survive. **KO** = -/- , **Het** = +/-, **WT** = +/+.

Table 4.2 Effect of various dosages of GPx1(-) and SOD1(-) in mice on 72 h survival of acetaminophen or diquat treatment.

Treatment	SOD1 Geno	GPx1 Geno	Survival Class	
AP	-/-	+/-	4 ± 0.39	A
	+/-	-/-	2.5 ± 0.23	AB
	+/-	+/-	4 ± 0.23	A
	+/-	+/+	3.5 ± 0.28	A
	+/+	+/-	2 ± 0.28	CB
DQ	-/-	+/-	1 ± 0.28	C
	+/-	-/-	1 ± 0.23	C
	+/-	+/-	4 ± 0.23	A
	+/-	+/+	4 ± 0.28	A
	+/+	+/-	4 ± 0.28	A
PBS	-/-	+/-	4 ± 0.32	A
	+/-	-/-	4 ± 0.28	A
	+/-	+/-	4 ± 0.28	A
	+/-	+/+	4 ± 0.32	A
	+/+	+/-	4 ± 0.32	A
	+/+	+/+	4 ± 0.21	A

Values are expressed as means ± SEM (n = 3-6). Values not sharing a common letter are significantly different at $P < 0.05$. Survival classes:
1 = <20 h; 2 = 20-40 h; 3 = 40-<72 h; 4 = >72 h.

had the steepest decrease in survival rate. Among AP treated mice, GPx1+/-|SOD1+/+ and GPx1-/-|SOD1+/- had the largest decrease in survival. A CPH model based on GPx1(-) copy number*SOD1(-) copy number*treatment (Figure 4.5) shows significant correlation with the experimental data but group sizes and survival rates preclude making statistically significant conclusions.

Biochemical Analyses

Plasma ALT: Time of death and treatment had significant effects on plasma ALT, a measure of liver toxicity. All mice that survived to the end of the experiment showed no significant change in ALT from PBS control. Mice who died in less than 20 hours of AP- or DQ- treatment showed significant increases in ALT compared with those that survived the injections (>72 h) and PBS control (3145 ± 877 and 2129 ± 537 versus 152 ± 368 U/L, respectively; $P < 0.05$). AP-treated mice who died between 20 - 40 h and 40 - >72 h had significant increases in ALT over control: (11740 ± 1074 and 6297 ± 1074 versus 152 ± 368 U/litre $P < 0.05$). DQ treated mice that died during the same time period had no significant ALT increases. (Figure 4.6).

GPx1 Activity

GPx1 genotype and SOD1 genotype both had significant effects on liver GPx1 activity. All GPx1+/- and GPx1-/- mice showed significantly lower liver GPx1 activity than GPx1+/+ mice (from 1 ± 32 to 553 ± 30 versus 1014 ± 36 U/mg protein, $P < 0.05$). Among GPx1+/- mice, those with SOD1-/- showed significantly lower liver GPx1 activity than those with SOD1+/- (364±40 versus 553 ± 30 U/mg protein, $P < 0.05$) and marginally lower activity than those with SOD1+/+ (364 ± 40 versus 504 ± 36 U/mg protein, $P=0.0778$). Treatment and survival time did not significantly affect liver GPx1 activity (Figure 4.7).

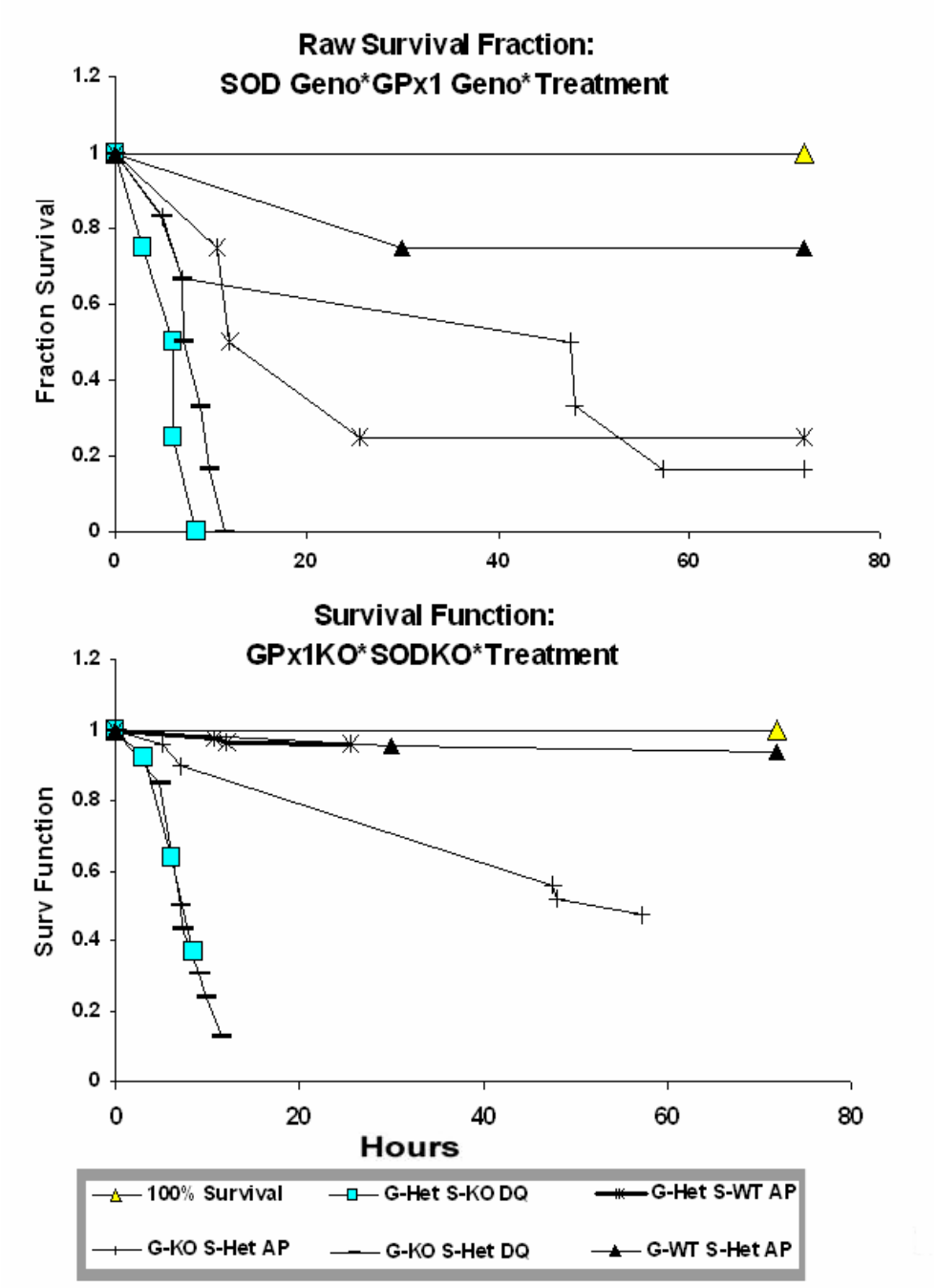

Figure 4.5. Seventy-two hour survival curves from experimental data and a Cox proportional hazards model of mice with various gene dosages of GPx1 and SOD1 treated with acetaminophen or diquat: GPx1, SOD1 and treatment effects. A Cox proportional hazards model that shows high correlation ($P < 0.05$) with experimental data is based on GPx1(-) copy number, SOD1(-) copy number and treatment. **S**=SOD1, **G=**GPx1, **KO** = -/- , **Het** = +/-, **WT** = +/+

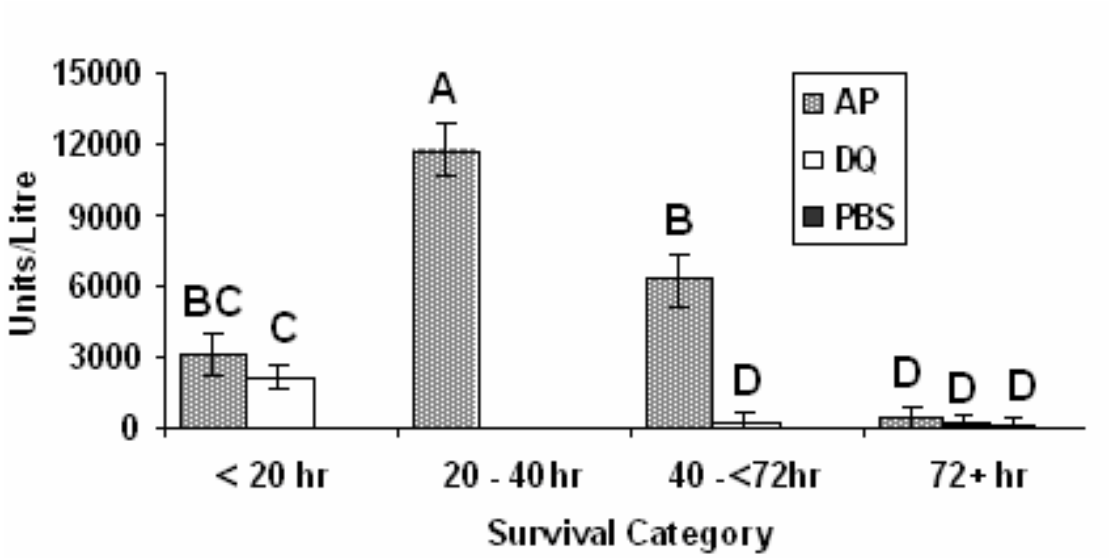

Figure 4.6. Plasma alanine aminotransferase activity in mice with various gene dosages of GPx1 and SOD1 treated with acetaminophen or diquat: 72 h survival study. ALT activity peaked at <20 h survival in DQ treated mice and at 20-40 h survival in AP treated mice. All mice that survived 72+ h had normal ALT activity. Values are expressed as means ± SEM (n = 9-24). Values not sharing a common letter are significantly different at $P < 0.05$.

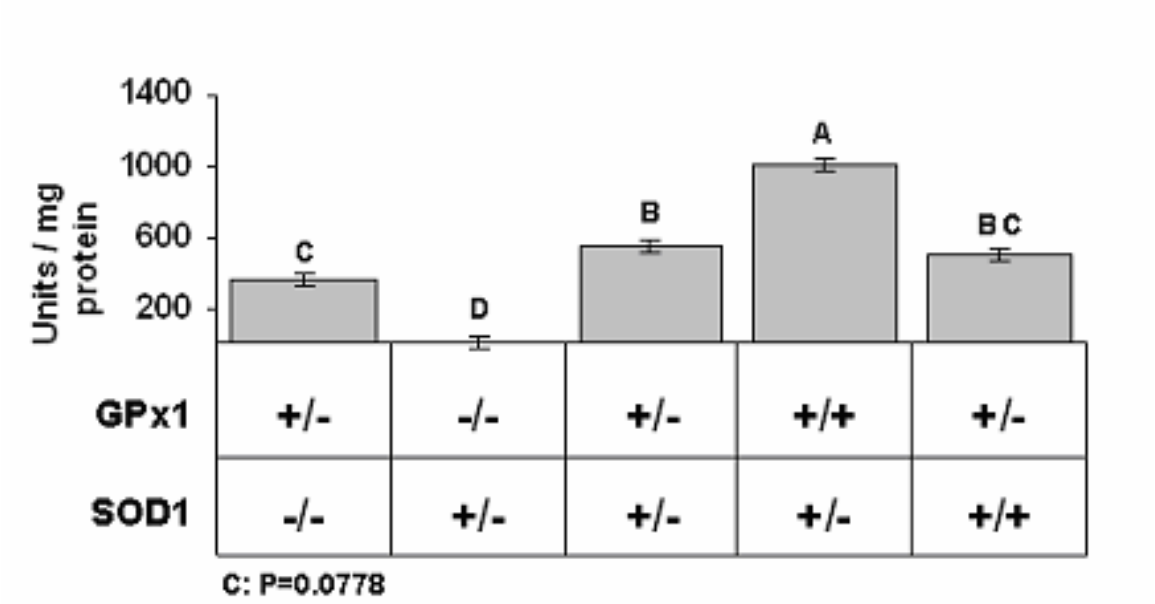

Figure 4.7. Liver GPx1 activity in mice with various gene dosages of GPx1 and SOD1: Gene dosage effects. GPx1-/- mice had negligible activity. All GPx1+/- had lower activity than GPx1+/+ mice. SOD1 genotype had an effect on GPx1 activity GPx1+/- mice had 34% and 28% reductions in liver GPx1 activity, depending on SOD1(-) copy number when compared with SOD1+/+. Values are expressed as means ± SEM (n = 7-16). Values not sharing a common letter are significantly different at $P < 0.05$. Values that share **C** are different at $P = 0.0778$.

A general linear model of liver GPx1 activity demonstrates this significant ($P < 0.01$) relationship between GPx1 activity and SOD1 genotype. Based on its R^2 value, this model accounts for over 89% of variability of liver GPx1 activity in this experiment (Figure 4.8).

SOD Activity

SOD1 genotype had the most significant effects on liver SOD activity. All SOD1 -/- mice had negligible activity (<25 units/mg protein) and PBS treated SOD1+/- mice had significantly lower activity than SOD1+/+ mice ($P < 0.05$, data not shown).

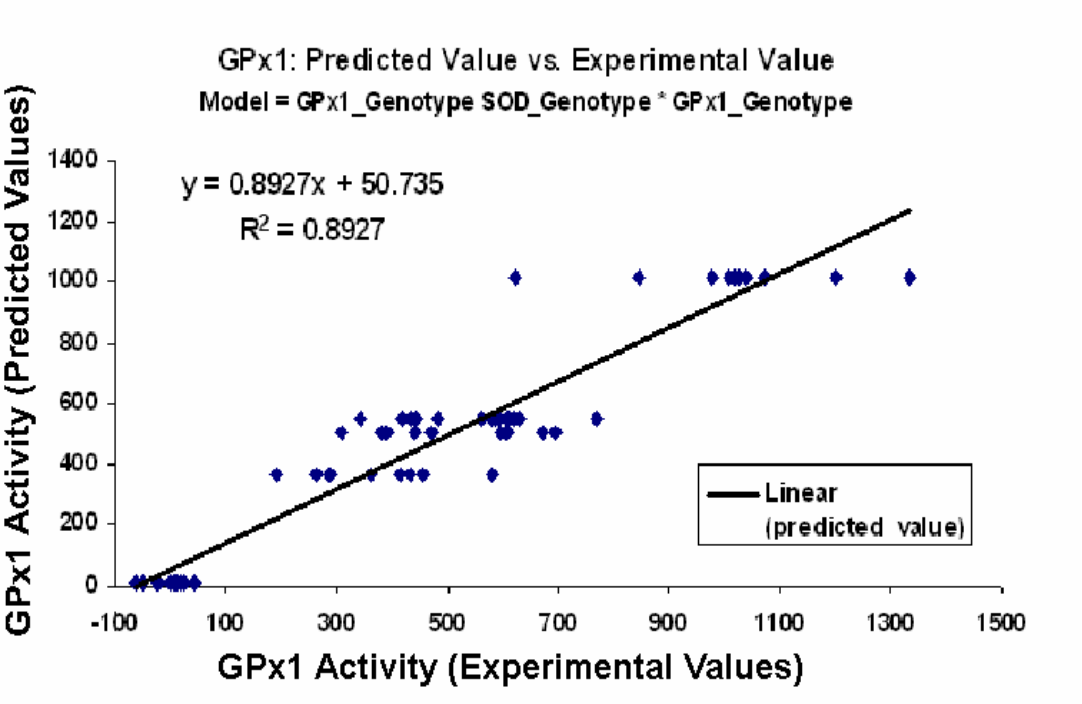

Figure 4.8. Correlation of general linear model prediction of GPx1 activity with experimental data based on GPx1 genotype and SOD1 genotype. A general linear model of liver GPx1 activity based on GPx1 genotype and GPx1*SOD1 genotype interaction is highly correlated with experimental GPx1 activity. This model accounts for nearly 90% of variation seen in the experimental data and describes a negative correlation between the SOD1(-) gene and GPx1 activity in the liver.

Discussion

This study has provided evidence of opposite effects of certain gene dosages of GPx1 and SOD1 in mice on survival of acetaminophen (AP) treatment and diquat (DQ) treatment. In this study, both raw survival curves based on experimental data and general linear models are flawed when GPx1 and SOD1 are considered separately. Small sample sizes and group survival rates preclude adjusting the effect of one gene for the effect of the other. We therefore believe that this has skewed some survival times and influenced interpretation of results. However, by use of stratified analyses, Cox proportional hazards (CPH) models were able to separate the survival curves of each gene. Sample size has also limited interpretation of CPH analysis since confidence intervals could not be calculated with such small numbers of observations. Primarily, we have based our analyses of individual gene effects on survival on correlation of CPH models with experimental data. General linear models provide some support for our interpretation of CPH models.

When the gene effects are separately analyzed in stratified CPH models, the data show that both SOD1-/- and GPx1-/- mice die rapidly with DQ treatment (<20 h). However, SOD1-/- mice survive AP treatment (>72 h) while GPx1-/- mice have a greatly decreased survival rate (majority die by 72 h). This shows the opposite effects that SOD1(-), but not GPx1(-), had on AP and DQ survival.

Opposite effects on DQ and AP survival were also seen when both GPx1 and SOD1 genotype were considered together. When treated with AP, mice with only one functional copy in total of GPx1 and SOD1 (GPx1+/-|SOD1-/- or GPx1-/-|SOD1+/-) survived the entire trial (>72 h). Conversely,

mice with the same genotypes died significantly earlier than control (<20 h) when treated with DQ.

When mice with only one allelic knockout in total of GPx1 and SOD1 (GPx1+/+|SOD1+/- or GPx1+/-|SOD1+/+) were examined, another interaction between these genes and treatments emerged. AP-treated GPx1+/-|SOD1+/+ mice died in less than 40 h ($P < 0.05$, compared with control) but AP-treated GPx1+/-|SOD1+/- survival time was not significantly different from control (>72 h). Since the difference between these genotypes is SOD1 copy number, SOD1(-) appears to have had a greater influence on AP survival than GPx1(-).

Our DQ treatment results are consistent with previous work on GPx1 knockout and SOD1 knockout mouse models (Cheng et al., 1998b; Ho et al., 1998) and the *in vivo* anti-oxidative functions of these proteins. However, our results are not entirely consistent with hypotheses of ROS and PN involvement in AP toxicity. It has been reported that AP metabolism produces superoxide and that exogenous SOD is able to protect against AP toxicity (Dai and Cederbaum, 1995; Nakae et al., 1990). Contrary to this, our results suggest that SOD1-/- mice, which have less protection against superoxide damage, are more resistant to AP toxicity. Since GPx1(-) increases sensitivity to AP toxicity, our results are consistent with PN production by AP and GPx1 participation in its detoxification (Hinson et al., 1998; Knight et al., 2001; Knight and Jaeschke, 2004). However, our findings are not consistent with the hypothetical role of superoxide in AP induced PN production. If it is assumed that SOD1-/- mice have a decreased ability to metabolize superoxide and therefore have a higher intracellular superoxide concentration, PN production and AP toxicity should be increased. Our experiments do not support this. Established hypotheses of AP toxicity may be incomplete or there may be

certain changes in AP metabolism related to SOD1(-) but not GPx1(-) which alter AP toxicity.

Aside from effects on survival, this study found a strong effect of SOD1(-) on GPx1 activity. When GPx1+/- mice with three different copy numbers of SOD1 were compared, we found that SOD1-/- mice had 72% ($P < 0.05$) and 66% ($P = 0.0778$) of SOD1+/- and SOD1+/+ liver GPx1 activity, respectively. Some work has indicated that GPx1 expression and activity can be regulated by changes in cellular oxygen tension (Cowan et al., 1993; Cao et al., 2003) and although it is reasonable to assume that SOD1(-) leads to these types of changes, we do not have evidence at this time to support such an hypothesis. Among PBS treated mice, SOD1 genotype was also highly correlated with liver SOD1 activity as follows: SOD-/- < SOD +/- < SOD +/+ ($P < 0.05$, data not shown).

We have used plasma ALT as an indicator of liver toxicity. Although there was not a highly significant effect of genotype on plasma ALT, treatment and survival time did have significant effects. According to models discussed above, treatment and survival time are related by genotype, therefore we believe genotype has an influence on these results. According to our ALT data, hepatocellular AP damage apparently peaks between 20 and 40 h while ALT of DQ-treated mice peaks at <20 h. Both AP and DQ liver injury recover to normal levels by 72 h. We suspect that these differences are due to the mechanisms of lethality of AP and DQ and how they may change over time. With a larger sample size, it would be interesting to outline the progression of ALT activity changes in these mice with various dosages of GPx1 and SOD1 in order to explore possible differences in lethality mechanisms. Earlier death (<20 h) may be due to loss of reducing equivalents (Cheng et al., 1999) while

later death (and ALT increases) may be due to hepatocellular injury (Cheng et al., 2003).

In summary, this study has shown that SOD1(-) is associated with decreased sensitivity to AP toxicity, increased sensitivity to DQ toxicity and decreased liver GPx1 activity. In contrast, GPx1(-) is associated with increased sensitivity to both AP and DQ toxicity. Based these results, we suggest that AP metabolism is changed in mice with SOD1(-). In addition, the temporal progression of hepatotoxicity may have been altered by changes in GPx1 and SOD1 gene copy number.

CHAPTER 5:

SUMMARY AND CONCLUSIONS

Knockout mouse models of antioxidant proteins provide a powerful tool to investigate the physiological functions of these proteins. Previously, work with whole animal research models on the selenoperoxidases and Cu,Zn-dependent superoxide dismutase was limited to nutritionally deficient models. These models could not be used to specifically address the effect of one protein without involvement of many other factors. Many questions remain to be addressed in whole animal models due to the limitations of these older methods. This thesis addresses the effects of altered gene dosage on antioxidant protein expression and sensitivity to xenobiotics.

The haploid insufficient model of GPx4 provides the first opportunity to independently test effects of reduced GPx4 activity. Selenium deficiency causes a number of physiological changes, including male reproductive dysfunction and increased pro-oxidant sensitivity that may be mediated through GPx4. Although a haploid insufficent model is not ideal for these investigations, it can provide insight into the physiological function of GPx4.

Previous work in our lab has shown that reduction of antioxidant enzyme activity is not always detrimental., We have found that GPx1 knockout decreased hepatocyte sensitivity to peroxynitrite (RNS) while increasing hepatocyte and whole animal ROS sensitivity (Cheng et al., 1998b; Fu et al., 2001). The GPx1 knockout-mediated changes in RNS sensitivity remained to be tested in whole animals. This question was expanded to include effects of another important antioxidant protein, SOD1, using knockout mouse models to specifically address the effect of various combinations of GPx1 and SOD1 gene dosage on ROS and RNS sensitivity and GPx1 and SOD activity.

Experiment 1 found that deletion of one copy of GPx4 resulted in a 24-39% reduction in GPx4 activities in liver, lung, kidney, and testes, compared with WT mice ($P < 0.05$). GPx1 activities in various tissues and GPx3 activities in plasma were unaffected by the GPx4 knockout therefore these selenoperoxidases have expression that is independent of GPx4.

The testes exhibited the highest percentage change in GPx4 activity in response to the gene knockout. However, since the knockout of one allele of *gpx4* did not lead to 50% activity reduction in the assayed tissues, as in some cases of gene knockout (Huang et al., 1996; Kline et al., 2002; Hattori et al., 2005), expression of the remaining allele may have been up-regulated to compensate for the deletion. In addition, testes showed the only significant change in tissue selenium concentration in GPx4+/- (34% reduction compared with WT mice, $P > 0.05$).

Experiment 1:

1. GPx4+/- mice had 24 - 39% reduction in GPx4 activity in lung, liver, kidney and testes
2. GPx4+/- did not significantly affect susceptibility to paraquat induced protein carbonyl formation
3. GPx4+/- did not affect GPx1 or GPx3 activity
4. GPx4+/- testis had a 34% reduction in selenium concentration

Experiment 2 provided evidence of opposite effects of certain gene dosages of GPx1 and SOD1 in mice on lethality of acetaminophen and diquat toxicity. Sample size has limited interpretation of statistical analyses but some significant effects and some general trends emerged. SOD1-/- and GPx1-/- mice both die rapidly with DQ treatment (<20 h, $P < 0.05$). However, SOD1-/-

mice survive AP treatment (>72 h) while GPx1-/- mice have a greatly decreased survival rate (majority die by 72 h), demonstrating opposite effects of SOD1(-), but not GPx1(-), on AP and DQ lethality.

Similar effects emerged when these two genes were considered in combination. Mice with only one functional copy in total of GPx1 and SOD1 (GPx1+/-|SOD1-/- or GPx1-/-|SOD1+/-) died significantly earlier than control (<20 h, $P<0.05$) when treated with DQ but survived the entire trial when treated with AP (>72 h).

Mice with only one knockout in total of GPx1 and SOD1 (GPx1+/+|SOD1+/- or GPx1+/-|SOD1+/+) had responses that further supported our hypotheses. AP-treated GPx1+/-|SOD1+/+ mice died significantly earlier than control (<40 h, $P < 0.05$) but AP-treated GPx1+/-|SOD1+/- survival was not significantly different from control (>72 h). Since a change in SOD1 copy number led to this difference, we speculate that SOD1(-) had a greater influence on AP survival than GPx1(-). The sensitivity of mice lacking functional GPx1/SOD1 copies to DQ treatment is consistent with previous work on GPx1 knockout and SOD1 knockout mouse models (Cheng et al., 1998b; Ho et al., 1998). Our results do not support those of Nakae et al., (1990) who reported that AP toxicity is partly due to superoxide production. Mice with SOD1(-) were more resistant to AP toxicity, even though they are less resistant to the superoxide generator, diquat. Since we found that GPx1(-) increased sensitivity to AP toxicity, our results are consistent with PN production by AP and GPx1 participation in PN detoxification (Hinson et al., 1998; Knight et al., 2001; Knight and Jaeschke, 2004) but do not provide supportive evidence.

A strong effect of SOD(-) on GPx1 activity was found. When mice with three different copy numbers of SOD1 were compared, liver GPx1 activity was reduced by as much as 34% compared with SOD1+/+ mice.

Finally, according to our ALT data, hepatocellular AP damage apparently peaks between 20 and 40 h while ALT of DQ-treated mice peaks at <20 h. All ALT activities returned to normal by 72 h. We suspect that these differences may be due to the mechanisms of AP and DQ lethality and how they change over time.

Experiment 2:

1. SOD1(-) made mice more susceptible to diquat lethality
2. SOD1(-) made mice less susceptible to acetaminophen lethality
3. GPx1(-) made mice more susceptible to both diquat and acetaminophen lethality
4. SOD(-) can rescue mice with GPx1(-) from acetaminophen lethality
5. SOD+/- and SOD -/- mice have 28% - 34% lower GPx1 activity
6. SOD(-) and GPx1(-) alter the progression and severity of diquat and acetaminophen hepatotoxicity

In conclusion, the deletion of one allele of GPx4 resulted in differential changes in GPx4 activities and selenium concentrations in lung, liver, kidney and testis. However, GPx4+/- mice showed no increased susceptibility to protein oxidation mediated by moderate oxidative stress and no apparent deficit in reproduction. SOD1(-) is associated with decreased sensitivity to AP toxicity, increased sensitivity to DQ toxicity and decreased liver GPx1 activity while GPx1(-) is associated with increased sensitivity to both AP and DQ toxicity. Based on these results, we suggest that AP metabolism and the

temporal progression of AP and DQ hepatotoxicity have been altered by changes in GPx1 and SOD1 gene copy number.

Future Directions of Research

The usefulness of the GPx4 haploid insufficient model is limited by its intact GPx4 allele. Taking Experiment 1 as an example, the function of the remaining GPx4 allele is sufficient for apparently normal physiological function. A full GPx4 knockout would be a very powerful model for research into its physiological role and embryonic lethality of the GPx4 nullizygous mouse may no longer be a limitation to production of this model. By avoiding a whole body knockout or delaying activation of the knockout until the mouse is mature, tissue specific and inducible gene knockouts based on the Cre/Lox system may provide a way to produce a true GPx4 knockout mouse (for review of Cre/Lox, see Sauer, 1998).

The production of a Cre/Lox GPx4 mouse in which the knockout can be induced once the animal has reached adulthood will be an important step forward in the study of GPx4 function. If this model is non-viable, cause of death alone would provide some important insight into the role of GPx4. In that case, testis, brain, liver, lung, kidney and muscle-specific GPx4 knockouts would be the alternate means of studying the still unclear physiological function of GPx4.

In Experiment 2, two very interesting observations stood out: 1) resistance to acetaminophen toxicity in SOD1 knockout mice and 2) decrease of GPx1 activity in SOD1 knockout liver.

The future of research into altered AP toxicity is in examining its mechanism. Is production of toxic metabolites changed? Is SOD1 directly involved in AP metabolism or do other changes account for this? Is the means

of detoxification of toxic metabolites changed? Could changes in superoxide concentration in the cell make those metabolites less toxic? Is clearance of the drug altered? How does SOD1 knockout affect drug clearance? These questions, and many more, open research possibilities in both drug metabolism and oxidative stress.

In the case of decreased GPx1 activity in SOD(-) mice, mechanism is again the key. If there is less GPx1, it may be due to increased degradation of GPx1 or a control on expression which is sensitive to its substrate, hydrogen peroxide. Oxidative damage to GPx1 due to altered cellular redox status may account for enhanced degradation. If the amount of GPx1 is unchanged, how is its activity being post-translationally regulated? Is this regulation sensitive to cellular redox status? Does this altered activity serve a physiological purpose? These avenues of research may lead in new directions for investigation of redox regulation of enzyme activity and the interdependency of antioxidant enzymes.

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